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HTLV-1 LATENCY-MAINTENANCE FACTOR p30<sup>II</sup> MODULATES  
TAX-INDUCED NF $\kappa$ B SIGNALING THROUGH A  
p53-REGULATED MECHANISM

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A Dissertation Presented to the Graduate Faculty of

Biological Sciences

Southern Methodist University

in

Partial Fulfillment of the Requirements

for the degree of

Doctor of Philosophy

with a

Major in Molecular and Cellular Biology

by

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May 18, 2019

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HTLV-1 Latency-maintenance factor p30<sup>II</sup> modulates  
Tax-induced NFκB signaling through a  
p53-regulated mechanism

Advisor: Professor Robert Harrod

Doctor of Philosophy conferred May 18, 2019

Dissertation completed April 8, 2019

The human T-cell leukemia virus type-1 (HTLV-1) infects CD4<sup>+</sup> T-cells and is the etiological agent of adult T-cell leukemia/lymphoma (ATL), an aggressive and often-fatal lymphoproliferative neoplastic disease. The major transactivator protein, Tax, drives proviral gene expression and replication and plays a key role during the early-stage immortalization of T-cells by HTLV-1. Tax has also been shown to regulate many host cellular signaling pathways, including CREB/ATF, SRF, and NFκB. Another accessory viral protein p30<sup>II</sup> acts as a latency-maintenance factor and negatively regulates proviral gene expression and replication. The p30<sup>II</sup> protein counters Tax-dependent gene expression at the transcriptional and posttranscriptional levels. HTLV-1 Tax constitutively activates the NFκB pathway. Intriguingly, the stathmin or OP18 (Oncoprotein-18) gene, a microtubule-destabilizing protein, is negatively regulated by p53/Sin3/HDAC1 repressor complexes and was recently shown to interact with and stabilize the p65<sup>RelA</sup> subunit of NFκB. Stathmin is associated with an aggressive disease phenotype in pancreatic cancers. Our lab has recently shown that p30<sup>II</sup> induces the expression of p53 dependent pro-survival genes such as TIGAR (*Tp53 induced glycolysis and apoptosis regulator*). In approximately half of all cancers, p53 is either mutated or functionally inactivated. However, in the majority of ATL patient isolates, the wildtype p53 protein continues to be expressed at high levels. My studies have demonstrated

that the HTLV-1 p30<sub>II</sub> protein inhibits Stathmin expression in Tax-expressing HTLV-1 infected cells and ultimately suppresses NFκB hyperactivation. My research studies have revealed that Stathmin/Op-18 might act as an important co-factor for NFκB-activation in HTLV-1 associated oncogenesis and highlights the cooperative role of the latency-maintenance factor, p30<sub>II</sub>, with the viral transactivator by preventing Tax-induced cytotoxicity and genomic instability and may allow for the continued proliferation of HTLV-1 transformed cells and conceivably aid in disease progression. Overall my research work reveals a mechanistic link between NFκB inflammatory signaling and genomic instability that may contribute to oncogenesis and transformation in certain human cancers.

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## LIST OF ABBREVIATIONS

ATL	adult T-cell leukemia/lymphoma
bZIP	basic leucine zipper
c-MYC	cellular avian myelocytomatosis viral oncogene homolog
CTL	cytotoxic T-lymphocytes
HAM/TSP	HTLV-1-associated myelopathy/tropic spastic paraparesis
HAT	histone acetyltransferase
HBZ	HTLV-1 basic leucine zipper factor
HTLV-1	human T-cell lymphotropic virus type 1
HR	homologous recombination
IDH	Infective dermatitis associated with HTLV-1
LTR	long terminal repeat
LUBAC	linear ubiquitin chain assembly complex
MT	microtubule
NEMO	NF $\kappa$ B essential modulator
NES	nuclear export sequence
NF $\kappa$ B	nuclear factor kappa B
NHEJ	non-homologous end joining
NLS	nuclear localization signal
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
ROS	reactive oxygen species
TIGAR	Tp53-inducible glycolysis and apoptosis regulator
TIP60	Tat-interacting protein kDa 60
TRE	Tax responsive elements

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# **CHAPTER I**

## **INTRODUCTION AND BACKGROUND**

### **1.1 Significance and hypothesis**

The human T-cell leukemia virus type-1 (HTLV-1) is an oncogenic retrovirus and is the etiological agent of adult T-cell leukemia/lymphoma (ATL) in humans. My dissertation focuses on an interplay between the cellular and viral proteins that could possibly lead to the development of ATL.

The viral transactivator and oncoprotein, Tax, is considered to play a central role in the process leading to ATL. Tax is a highly promiscuous viral protein containing a number of interesting domains allowing it to interact with a myriad of cellular factors and thus affecting a large number of cellular functions. Tax is also a potent activator of a variety of transcription pathways and has been shown to be sufficient to immortalize T-cells in vitro and thus plays an important role in cellular transformation. Persistent activation of NF $\kappa$ B by Tax is one of the characteristic features of ATL. Tax immortalizes primary human T-cells in a manner highly dependent on its ability to activate NF $\kappa$ B. However, a complete mechanism for Tax-mediated oncogenesis remains to be fully elucidated. The expression of Tax alone has been shown to be cytotoxic and also induces apoptosis and senescence. One possibility is that the other viral genes of pX region cooperate with Tax. HTLV-1 p30<sup>II</sup> is a latency-maintenance factor which negatively regulates HTLV-1 replication and counters Tax-dependent gene expression at the transcriptional and posttranscriptional levels. Previous studies from our lab have shown that p30<sup>II</sup> cooperates with

cellular oncoproteins and induces aberrant lymphoproliferation through the activation of p53-dependent pro-survival genes. The p53 gene is mutated or functionally inactivated in nearly half of all cancers, however, in HTLV-1-induced ATL the p53 gene is usually wild-type. Interestingly, Stathmin – a p53 regulated protein has been reported to act as a cofactor for NFκB signaling by stabilizing the p65<sup>RelA</sup> protein in aggressive pancreatic cancers. Stathmin is a microtubule destabilizer which stimulates MT transition from elongation to shortening and is referred to as a catastrophe promoter. HTLV-1 Tax oncoprotein which activates NFκB signaling is also responsible for DNA damage and genomic alterations. This led us to investigate the p65<sup>RelA</sup> and Stathmin molecular interactions with regard to Tax-induced NFκB hyperactivation and genomic instability.

*The objective of my dissertation research is to discover the molecular mechanism(s) for how HTLV-1 p30<sup>II</sup> and host cellular factors p65<sup>RelA</sup> and Stathmin may modulate Tax-induced NFκB activation and promote tumorigenesis during HTLV-1-induced carcinogenesis.*

## **1.2 HTLV-1 AND ATL**

Adult T-cell Leukemia/Lymphoma (ATLL/ATL) was first described as a new type of adult onset T-cell leukemia by Uchiyama et al in 1977 even before the discovery of Human T-cell lymphotropic virus (HTLV-1). One of the striking aspect of the disease was epidemiological clustering of affected individuals in certain areas of Japan, indicating towards involvement of a transmissible agent in this disease (Uchiyama et al. 1977). ATL spectrum was divided into five types depending on the clinical features: pre-ATL, chronic, smoldering, acute T- cell leukemia and lymphoma-type. The acute form of ATL comprises 55–75% of all ATL, with chronic and cutaneous forms comprising the remaining 25% (Takatsuki et al. 1985; Yamaguchi et al. 1983; Yamaguchi & Takatsuki 1993).

Human T-cell lymphotropic virus (HTLV-1) was the first infectious human retrovirus reported to be the etiological agent of cancer and was first isolated in a patient with cutaneous T-cell lymphoma and was soon correlated with ATL (Poisez et al. 1980; Yoshida et al 1982). Two years later, HTLV-2 was identified in a patient diagnosed with hairy cell leukemia (Kalyanraman VS et al. 1982). Over years, HTLV-1 has been deemed to be more clinically significant of the two and has been proven to be the etiological agent of multiple disorders including two major diseases: adult T-cell leukemia/lymphoma (ATLL) and tropical spastic paraparesis/HTLV-1-associated myelopathy (Osame M et al. 1986). Other conditions associated with HTLV-1 infection are uveitis, rheumatoid arthritis/Sjogren syndrome, bronchioalveolar pneumonitis, autoimmune thyroiditis and infective dermatitis (Manns A et al. 1999; Jones K.S. et al. 2008; LaGrenade L et al. 1990). HTLV-1 is a complex deltaretrovirus that infects and transforms CD4+ T-lymphocytes. It is found in clusters of endemic infection in parts of Southeast Asia, including Japan, Taiwan, Malaysia, Africa, Caribbean and parts of Central and South America, the Middle East, and Melanesia (Poisez et al. 1980; Matsuoka M et al. 2005). There are about 10-20 million people estimated to be HTLV-1 carriers worldwide (Gessain A et al. 2012). Almost 90% of carriers remain asymptomatic throughout their lifetime and only 3-5% individuals develop disease with the average age of clinical onset between 40-60 years (Datta et al. 2007; Matsuoka 2003; Green et al. 2001). The cumulative risks of developing ATL among HTLV-1 carriers are approximately 6.6% for males and 2.1% for females (Proietti FA et al. 2005).

### *Viral Transmission and Infectivity*

The most important route of HTLV-1 transmission is from mother to child, predominantly through breastfeeding. It has been shown that approximately 10-25% of breast fed infants of HTLV-1 positive seropositive mothers are also seropositive (Gessain A and Connor O. 2012).

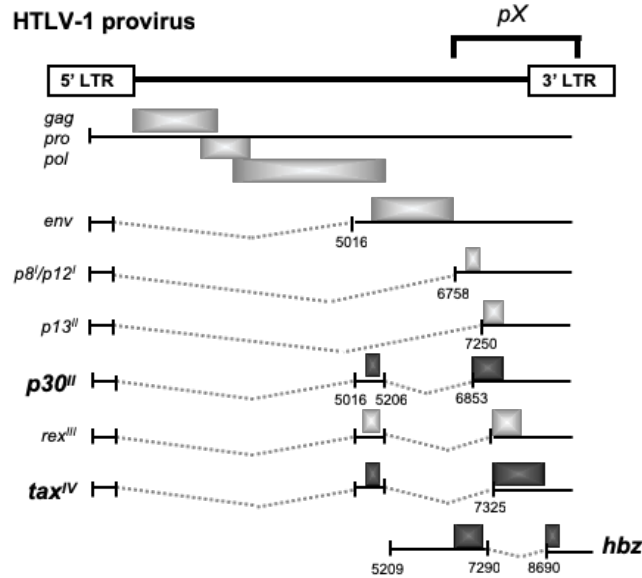


There is also a <3% risk of HTLV-1 vertical transmission from mother to child through transplacental route or during delivery. It is also transmitted horizontally by sexual course and through blood transfusion (Carnerro-Proietti AB et al. 2014). Since the virus by itself is very poorly infectious, effective transmission requires live infected T-lymphocytes such as T-cells in breast milk, in semen and in blood of HTLV-1 carriers (Taylor GP et al. 2005). Viral infectivity is primarily cell to cell and is mediated through a virological synapse. In the incidence of contact between an infected and uninfected cell, polarization of microtubule organizing center (MTOC) occurs at the cell to cell junction and a virological synapse is formed (Igakura T et al. 2003). Following the synapse formation, HTLV-1 Gag complex and viral genomic RNAs accumulate and are sent to the uninfected cell. HTLV-1 utilizes the ubiquitous vertebrate glucose transporter Glut-1, as well as neuropilin (NPR1) and surface heparin sulfate proteoglycan receptors to infect and transform their primary target, that is, CD4+ T-helper lymphocytes (Manel et al. 2003; Takenachi N et al. 2007). Later it was shown to also infect other immune substrates including CD8+ lymphocytes, CD14+ macrophages, and dendritic cells. More recently, it was shown that HTLV-1 virions can efficiently infect dendritic cells, and these cells can then rapidly infect CD4+ T cells (Lepoutre et al. 2009).

#### *HTLV-1 Proviral Genome*

The HTLV-I virus was the first human retrovirus to be identified and was initially isolated in 1978 by Gallo and Poiesz from the T lymphocytes of a patient with a cutaneous T-cell lymphoma (Poiesz BJ et al. 1980; Gallo RC et al. 1982).

HTLV-1 is a virus with a single-stranded RNA genome of approximately 9 kb. The viral genome encodes structural and enzymatic proteins and tandem long terminal repeats (LTRs) at the 3' and 5' end of the genome which are divided into unique region 3' (U3), repeated region (R),



**Figure 1. HTLV-1 proviral genome and its products.** HTLV-1 has four genomic regions namely gag, pol, env and a unique pX region that encodes its regulatory and accessory genes. hbz is encoded from the minus strand of the mRNA (Malu et al; 2019, submitted)

unique region 5' (U5) that allow the proviral transcription through the U3 region as well as mRNA termination, polyadenylation and strand transfer during reverse transcription (Zhao LJ and Giam CZ 1992; Lairmore et al. 2011; Green et al. 2001; Johnson et al. 2001). Moreover, there are three imperfect twenty-one base pair repeats termed Tax Responsive Elements or TREs which are necessary for transcriptional activation by the Tax protein and also act as an active site for chromatin remodeling (Lairmore et al. 2011). Similar to other retroviruses, HTLV-1 has the structural proteins group-specific antigen (gag), protease (pro)/polymerase (pol), and envelope (env) genes are coded from unspliced/singly spliced mRNAs (Lee TH et al. 1984). The Gag (p55) polyprotein is cleaved by viral proteases into Capsid (p24), Nucleocapsid (p15), and Matrix (p19), which are required for packaging (Franchini GV. 1995). In addition to the structural genes, the

HTLV-1 genome contains a unique pX region at the 3' end, which encodes regulatory proteins. The two regulatory genes tax and rex are encoded by open reading frames (ORF) IV and III, respectively, and are synthesized from a doubly spliced mRNA. The pX ORFs I and II produce alternatively spliced forms of mRNA, which encode four accessory proteins, p12<sup>I</sup>, p27<sup>I</sup>, p13<sup>II</sup>, and p30<sup>II</sup>. HBZ is encoded on the minus strand of the provirus and transcribed from the 3' LTR. Other viral genes are transcribed as sense transcripts from the 5' LTR. Transcription from the 5' LTR is highly inducible by Tax, in which CREB and p300/CBP are involved (Nyborg JK et al. 2010).

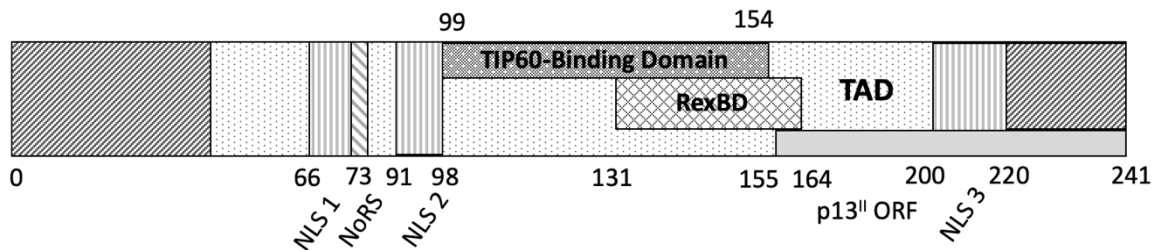
*Orf-I (p8/p12):* The orf-I products are 2 accessory proteins p12 and p8 made from singly-spliced mRNA. HTLV-1 infected individuals exhibit a cytotoxic T-lymphocyte (CTL) response towards orf-I products. Proteolytic cleavage of p12 protein precursor removes a non-canonical endoplasmic reticulum (ER) retention/retrieval signal at its amino-terminus to yield the p8 protein (Fukumoto R et al. 2009). The orf-I mRNA has been detected in all ATL patient isolates (Ding et al, 2002; Edwards et al, 2011). The 12-kD precursor protein (p12), is a 99 amino acid long hydrophobic protein which resides in the endoplasmic reticulum (ER)/Golgi (Johnson JM et al. 2001; Koralnik IJ et al. 1993). The HTLV-1 virus usually maintains lifelong infection once an individual is a carrier, and in doing so it constantly evades the host immune responses. One such mechanism is decreased expression of major histocompatibility complex (MHC) class I, intercellular adhesion molecule 1 (ICAM-1) and ICAM-2, on cell surface and protecting the CD4<sup>+</sup> HTLV-1–infected cells by natural killer cells. p12 binds to the heavy chain of MHC class I in the ER and sends it to cytosol for proteasome mediated degradation thus inhibiting recognition of infected cell by the CTLs (Johnson JM et al. 2001). In the ER, p12 protein interacts with the interleukin-2 receptor (IL-2R)  $\gamma$  and  $\beta$  chains (Mulloy et al. 1996) and increases signal transducer and activator of transcription 5 (STAT5) phosphorylation leading to an increase in STAT5

activation (Van Prooyen N et al. 2010; Nicot C et al. 2001) and increases the production of IL-2 (Nicot C et al. 2001; Ding W et al. 2003). p12 also causes an increase in release of calcium and calcium-dependent proteins (calnexin and calreticulin; ER proteins that regulate calcium storage) from the ER, and also results in activation of Nuclear Factor of Activated T-cells (NFAT), a cellular transcription factor in order to promote T-cell activation and proliferation. This increase in intracellular calcium levels and calcium-dependent signaling during early infection not only promotes proliferation but can also trigger other cellular pathways, including those important for viral transmission. Both these functions of p12 further underscore its importance in T-cell activation (Ding et al, 2001; Albrecht et al, 2000; Van Prooyen et al, 2010). Altogether, the ER-associated functions of p12 seem to promote the proliferation of infected T-cells and protect them from host immune recognition.

The loss of the p12 N-terminus allows p8 protein to go to cell membrane lipid rafts (Van Prooyen N et al. 2010). The p8 protein is recruited to immunological synapse by trafficking to lipid rafts at the site of TCR ligation and downregulates TCR proximal signaling through decreased phosphorylation of Linker of Activation for T-cells (LAT) (Fukumoto R et al. 2009) and increases lymphocyte function-associated antigen-1 (LFA-1) clustering by colocalization (Van Prooyen N et al. 2010). Furthermore, p8 has been shown to have the ability to be transmitted from cell-to-cell through cellular conduits and it increases HTLV-1 transmission by increasing T-cell adhesion and formation of cellular conduits (Van Prooyen N et al. 2010).

*Orf-II (p30<sup>II</sup>/p13<sup>II</sup>)*: The orf-2 encodes for two proteins p30<sup>II</sup> and p13 which are produced through alternative splicing. The larger protein, p30<sup>II</sup>, is encoded by a doubly-spliced message including the first and second exon of Tax spliced to the acceptor site, whereas the smaller protein, known as p13, contains the C-terminal 87 amino acids of p30<sup>II</sup> and is produced from a singly-

spliced message by splicing of the first Tax exon directly to the splice acceptor at position 6875 (Ciminale V et al. 1992). CTLs and antibodies for ORF-II products have been isolated from HTLV-1-infected carriers and ATL patients (Chen et al. 1997; Pique et al. 2000; Lairmore et al, 2011). ORF-II products have been shown to be necessary to maintain a high proviral load and to allow for the development of latency and disease (Silverman et al, 2004; Lairmore et al, 2011). Bartoe et al showed that ablation of p30<sup>II</sup> expression resulted in a significant decrease in HTLV-1 proviral load in rabbit model (Bartoe JT et al. 2000). The p30<sup>II</sup> protein is intriguing because it is reported to act as both a transcriptional and posttranscriptional regulator; a characteristic shared by another nucleolar resident protein, nucleolin (Ciminale et al, 1992).



**Figure 2. Diagram of HTLV-1 p30<sup>II</sup> accessory protein and its functional domains.** HTLV-1 p30<sup>II</sup> contains a TIP60-binding domain (aa 99-154) and a Rex binding domain (RexBD) (aa 131-164). TAD: transcription activation domain, NLS: nuclear localization domain, NoRS: nucleolar retention signal. The p13<sup>II</sup> peptide is encoded by the C-terminal domain (aa 155-241).

The p30<sup>II</sup> protein also known as Tax ORF-II (tof-II) is a 241 amino acid long protein which is highly basic and has a net positive charge. This protein contains three nuclear localization signals (NLS1, NLS2, and NLS3) and an arginine-rich nucleolar localization/retention (NoRS) domain (Ghorbel S et al. 2006). In addition, p30<sup>II</sup> also contains a Rex-binding domain (RexBD), a p30<sup>II</sup>-binding domain, and a DNA-binding domain (Baydoun HH et al. 2008). The p30<sup>II</sup> protein localizes

within the nucleus and nucleolus. It is specifically located in a granular component which is the site of assembly of ribosome subunits and de novo mRNA production. This localization explains the ability of p30<sup>II</sup> to bind the 60s ribosomal subunit protein L18a and to retain the newly transcribed tax/rex mRNA in the nucleus (Ghorbel S et al. 2006). p30<sup>II</sup> promotes virus latency by retaining tax/rex mRNA within the nucleus to prevent its export to the cytoplasm, and by downregulating Tax and Rex production p30<sup>II</sup> further plays a role in suppressing viral replication (Nicot C et al. 2004). In instances of DNA damage, p30<sup>II</sup> interferes with DNA repair processes by specifically delocalizing from the nucleoli to the nucleoplasm (Baydoun HH et al. 2011). The localization of p30<sup>II</sup> within the nucleus and nucleolus suggests its possible role in mediating critical cellular processes such as cell cycle progression, DNA repair, and mRNA export. Another hypothesis suggests that p30<sup>II</sup> retention within nucleoli may serve as a reservoir where it is stored until the protein is needed in the nucleus (Baydoun HH et al. 2011).

Post-transcriptional Regulation: The p30<sup>II</sup> protein controls viral gene expression at post-transcriptional level by binding to the tax/rex mRNA at the p30<sup>II</sup> mRNA-responsive element (p30<sup>II</sup>RE) and also to Rex at the RexBD (Sinha-Datta U et al. 2007). Rex and p30<sup>II</sup> are antagonistic to each other since Rex plays a role in nuclear-cytoplasmic export of unspliced and singly-spliced mRNA while p30<sup>II</sup> actually retains the tax/rex mRNA transcripts in the nucleus. Rex usually binds to the Rex responsive elements (RexRE) at the 3' end of viral mRNA and shuttles unspliced gag/pol and singly spliced env transcripts to the cytoplasm. p30<sup>II</sup> interacts with Rex to inhibit Rex-mediated nuclear export of double spliced viral mRNA, including tax transcripts. Rex is not accessible to p30<sup>II</sup> when it is bound to mRNA transcripts and is able to shuttle transcripts to the cytoplasm. However, viral mRNA-bound p30<sup>II</sup> still retains tax/rex transcripts in the nucleus (Sinha-Datta U et al. 2007). This nuclear retention of viral mRNA is reversed by an excess of Rex,

which displaces p30<sup>II</sup> from the p30<sup>II</sup>RE. By decreasing the translation of tax/rex mRNA; the two positive regulators of viral replication, p30<sup>II</sup> promotes latency allowing the virus infected cells to escape host immune surveillance and facilitates propagation through cell division and clonal expansion of infected cells (Silverman et al. 2004; Edwards et al. 2011). Also, it was speculated that this interplay between p30<sup>II</sup> and Rex can play an important part in establishing viral latency (Sinha-Datta U et al. 2007). Furthermore, microarray studies showed that p30<sup>II</sup> is also capable of regulating cellular gene expression at post-transcriptional levels. It was seen that in the presence of p30<sup>II</sup> there was a decrease in cytoplasmic expression of genes involved in cell signaling, transcription, translation, replication, cytoskeleton and metabolism while an increase in genes involved in DNA damage repair, apoptosis and cell adhesion (Taylor et al, 2009).

**Transcriptional Regulation:** Owing to its nuclear localization signals and serine- and threonine-rich regions, p30<sup>II</sup> shows distant homologies with the activation domain of cellular transcription factors Oct-1, Oct-2, Pit-1, and POU-M1, p30<sup>II</sup> was proposed to have a transcriptional function (Ciminale et al, 1992; Edwards et al, 2011; Taylor et al, 2009; Albrecht and Lairmore, 2002). It was shown that the p30<sup>II</sup> protein colocalizes with Tax in cell nuclei and regulates gene expression by binding to the KIX domain of CBP/p300 (Zhang et al, 2001). Furthermore, p30<sup>II</sup> was able to disrupt CREB-Tax-CBP/p300 complexes bound to the viral 21-bp repeats (Michael B et al. 2006). Altogether, these data suggest that p30<sup>II</sup> acts as a repressor of transcription by sequestering CBP/p300 from the pool of available transcription factors. Therefore, at higher concentrations p30<sup>II</sup> may serve to promote viral persistence by reducing viral gene expression and thus reducing immune recognition of infected cells (Zhang et al., 2000).

Through microarray analyses by various groups, p30<sup>II</sup> has been shown to alter the expression of a variety of gene families including those that have a role in transcription, translation, cell cycle

progression, DNA replication and repair, cell signaling, angiogenesis, cell migration, and apoptosis (Taylor M et al, 2009; Edwards et al, 2011; Awasthi S et al, 2005). p30<sup>II</sup> also selectively activates genes involved in T cell signaling/activation and enhances transcription mediated by NFAT, NFκB, and AP-1 (Michael et al, 2004). The ability of p30<sup>II</sup> to transcriptionally activate cellular genes was reported in a study where p30<sup>II</sup> binds to and stabilizes the Myc/TIP60 transcriptional complex (Awasthi S et al., 2005). Furthermore, focus-formation assays showing that p30<sup>II</sup> cooperated with Myc allude to p30<sup>II</sup>'s role in transformation by enhancing Myc-responsive genes (Awasthi S et al., 2005). The p30<sup>II</sup> protein contains a histone acetyltransferase TIP60-binding domain spanning amino acids 99-154. Through this domain, p30<sup>II</sup> interacts with cellular TIP60 and enhances cMyc associated transcriptional activity on the cyclin D2 promoter leading to increase in cell-cycle progression and increased cellular transformation (Awasthi S et al, 2005). It was seen that p30<sup>II</sup> is present in c-MYC-containing nucleoprotein complexes in HTLV-1-transformed T-lymphocytes. Moreover, p30<sup>II</sup> leads to inhibition of apoptosis in proliferating cells expressing c-MYC under conditions of genotoxic stress. These reports provide new insight into the mechanism of cooperation between p30<sup>II</sup> and c-MYC and demonstrate its possible cooperation with retroviral accessory proteins during HTLV-1-associated T-cell transformation (Awasthi S et al, 2005; Romeo MM et al, 2015). Furthermore, p30<sup>II</sup> can interact with the PU.1 transcription factor through its DNA binding domain leading to a decrease in the DNA binding ability of PU.1. The PU.1 directly regulates cell surface expression of TLR4 and in presence of p30<sup>II</sup> expression there is suppression of TLR4 leading to a significant decrease in pro-inflammatory cytokines TNF-α, MCP-1 and IL-8 and an increase in the expression of anti-inflammatory cytokine IL-10 (Datta et al, 2006). The p30<sup>II</sup> protein cooperates with Tax and HBZ and enhances their oncogenic potential in colony transformation/foci-formation assays. Hutchison



et al showed that the HTLV-1 latency-maintenance factor p30<sup>II</sup> by inducing the TP53-induced glycolysis and apoptosis regulator (TIGAR) promotes immune evasion and survival of HTLV-1 infected cells by countering the oxidative stress, mitochondrial damage, and cytotoxicity caused by the viral oncoproteins Tax and HBZ. They further demonstrated that p30<sup>II</sup> cooperates with the viral transactivator to promote oncogenic colony formation in vitro, by activating p53 dependent pro-survival signals including the TIGAR and suppresses Tax-induced oxidative stress (Hutchison T et al. 2018). Overall, these studies indicate that p30<sup>II</sup> regulates transcription of viral genes as well as cellular genes and also regulates innate and adaptive immunity through influencing cellular transcription, to create an environment that promotes viral gene expression and survival of infected cells.

**Cell-cycle Regulation:** Cell-cycle control is a highly regulated pathway that ensures proper cellular division and cell-cycle progression. Some studies have shown that p30<sup>II</sup> activates the G2/M checkpoint by increasing the phosphorylation of Chk1 and subsequently the phosphorylation of Cdc25 (Datta et al. 2007). p30<sup>II</sup> binds to and recruits TIP60 to Myc-responsive promoters stabilizing the Myc/TIP60 interactions. The stabilization of Myc/TIP60 interactions by p30<sup>II</sup> causes transactivation of the cyclin D2 promoter and progression to S-phase leading to polyploidy and enhanced Myc-transforming activities (Awasthi et al, 2005). However, Baydoun et al showed that p30<sup>II</sup> delays S-phase entry by binding to Cyclin E and Cdk2. This interaction prevents the formation of active Cyclin E/Cdk2 complexes causing a decrease in Rb phosphorylation and preventing the release of E2F, ultimately delaying the start of S-phase (Baydoun et al, 2011).

**DNA damage repair:** Retrovirus integration leads to double stranded DNA damage response where the DNA double strand breaks are highly toxic, and the cells must repair DNA damage in

order to avoid apoptosis. Double-strand DNA break repair can be carried out by either homologous recombination or non-homologous end joining (NHEJ). Under conditions of double-strand DNA breaks, the cell activates ATM (ataxia telangiectasia mutated) leading to the autophosphorylation of ATM followed by the phosphorylation of H2A.X, and finally activation of Checkpoint kinase 2 (chk2) (Anupam et al, 2011; Baydoun et al, 2011). Chk2 phosphorylates p53, stabilizing p53 and preventing p53 turnover by MDM2, thereby allowing for the repair of the breaks or the induction of apoptosis (Baydoun et al, 2011). p30<sup>II</sup> down-regulates ATM and favors non-homologous end joining (NHEJ). p30<sup>II</sup> interacts with REG $\gamma$  - a nuclear proteasome activator, and targets ATM for proteasome mediated degradation. Lowering the levels of ATM upon DNA damage decreases p53 mediated apoptosis and promotes the survival of infected cells (Anupam et al. 2011). Hence, by preventing apoptosis, p30<sup>II</sup> confers a growth advantage to those cells with DNA-damage. p30<sup>II</sup> also translocates from the nucleolus to the nucleus upon DNA damage and inhibits homologous recombination while promoting non-homologous recombination. p30<sup>II</sup> inhibits homologous recombination by disrupting the MRE11/Rad50/NSB1 (MRN) complex and pushing the cell to instead utilize NHEJ which allows for more mutations (Baydoun et al, 2011).

The second product of HTLV-1 orf-II is the singly spliced 87 amino acid long protein made from the C-terminus of p30<sup>II</sup>, known as p13<sup>II</sup> (Albrecht and Lairmore, 2002; Ciminale et al, 1992). p13<sup>II</sup> is localized in the inner mitochondrial membrane owing to its special N-terminus mitochondrial-targeting signal (Edwards et al, 2011; Albrecht and Lairmore, 2002). p13 forms an amphipathic  $\alpha$ -helix across the inner mitochondrial membrane which triggers an inward K<sup>+</sup> and Ca<sup>+</sup> current causing mitochondrial membrane depolarization and activation of the electron transport chain and enhancing reactive oxygen species (ROS) production (Silic-Benussi et al, 2010). When expressed at high levels, p13 localizes within the nucleus. In the presence of Tax,

p13 is ubiquitinated and translocates to nuclear speckles (Andresen V et al, 2011). In the nucleus, p13 decreases transcription of cellular and viral genes by inhibiting formation of Tax-CBP/p300 complex. p13 also increases the sensitivity of T-cells to pro-apoptotic stimuli such as Fas ligand (FasL), C2 ceramide, and glucose deprivation (Andresen V et al, 2011; Hiraragi H et al, 2005). This pathway also triggers translocation of Ras to mitochondria and requires Ras farnesylation. Farnesylated Ras goes to mitochondria and directly binds Bcl-2 inhibiting the anti-apoptotic effects of Bcl-2. (Hiraragi H et al, 2005). This effect is interesting because p13 was also shown to bind to farnesyl pyrophosphate synthase which is an enzyme required for the synthesis of precursors necessary for the farnesylation of Ras and other substrates (Lefebvre et al, 2002). Localization of the p13 protein within mitochondria and the nucleus suggests that this protein may modulate effects on apoptosis and transcriptional regulation.

*Orf-III p21<sup>Rex</sup> and Rex (p27)*: Two products p21<sup>Rex</sup> and Rex (p27) are formed from doubly spliced bicistronic mRNA from orf-III. The major Rex open reading frame (Rof) protein is Rex however the splice variant p21<sup>Rex</sup> is also produced at detectable levels (Nakano and Watanabe, 2012; Koralnik et al, 1993; Baydoun et al, 2008). CTLs against both these proteins have been detected in HTLV-I infected asymptomatic carriers, HAM/TSP and ATL patients (Pique et al, 2000).

HTLV-1 Rex is a major post-transcriptional regulator of viral expression. It is responsible for active viral replication in the early phase of infection and for reduction of viral activity to establish latency in the late phase of infection (Nakano and Watanabe, 2012). Rex is a 27kD phosphoprotein that binds to and facilitates nuclear export of viral mRNA. Rex also binds to and exports unspliced and singly spliced mRNA coding for viral structural protein. This critical nuclear-cytoplasmic export function and post-transcriptional control of mRNA translation makes Rex indispensable for

viral replication (Inoue et al. 1987; Hidaka et al. 1988; Gröne et al. 1996). Rex binds to the Rex responsive element (RxRE) of the viral transcript with high affinity. The 255 nucleotides long RxRE sequence is located in the U3 to R region of the 3' LTR and forms a stable secondary structure consisting of four stem loops (Ahmed et al., 1990). Since RxRE locates to the U3 and R regions, all unspliced, singly spliced and doubly spliced HTLV-1 mRNA have RxRE. RxRE allows Rex to selectively bind to unspliced and partially spliced HTLV-1 mRNA in the nucleus and exporting them to the cytoplasm, preventing further splicing and enhancing their effective translation (Hidaka et al, 1988; Adachi et al, 1990, 1992; Hamaia et al, 1997). Rex also has a very basic arginine-rich N-terminal RNA binding domain which also serves as nuclear localization signal (NLS) and as binding domain for p30<sup>II</sup> (Nakano and Watanabe, 2012; Baydoun et al, 2008; Bai et al, 2012). This RNA binding domain allows for binding to the Rex responsive element and additionally it allows for Rex to be imported into the nucleolus (Baydoun et al, 2008). Through the leucine-rich nuclear export signal (NES) Rex binds to Exportin-1 (CRM1), a cellular nuclear export protein, to facilitate Rex shuttling between the nucleus and cytoplasm (Nakano and Watanabe, 2012; Baydoun et al, 2008; Bai et al, 2012). NES is very hydrophobic and is also referred to as the Rex-activation domain. Through interactions with CRM1, NES can induce Rex multimerization (Bai et al, 2012; Baydoun et al, 2008). Rex multimers are assembled at the RxRE enabling Rex nuclear export (Bai et al, 2012; Baydoun et al, 2008, Nakano and Watanabe, 2012). Rex also increases the expression of il-2 $\alpha$  mRNA, although the mechanism through which it upregulates is not clear. IL-2R $\alpha$  overexpression in HTLV-1-infected cells increases the cellular response to IL-2. Rex is capable of stabilizing il-2 $\alpha$  mRNA up to fivefold (Nakano and Watanabe, 2012); this explains the overexpression of this gene in HTLV-1-infected and ATL cells, at least

partly, by the function of Rex. Altogether it appears that Rex functions as a positive regulator of viral gene expression and helps regulate the switch from viral replication to latency.

The N'-truncated form of p27Rex is p21Rex. p21Rex lacks 78 aa of the N-terminus region, including RBD/NLS and the N'-multimerization domain (Kiyokawa et al., 1985). The p21Rex transcripts are expressed in HTLV-1-infected cell lines and have also been detected in primary peripheral blood mononuclear cells from HTLV-1 carriers and ATL patients (Orita et al, 1992). It is hence speculated that p21Rex plays a role in the HTLV-1 life cycle as a dominant negative form of p27Rex.

*HBZ*: HBZ was the first viral protein found to be encoded in the antisense ORF of HTLV-1. There are two transcriptional isoforms of the HBZ: an unspliced (usHBZ) form and a spliced (sHBZ) form. usHBZ was discovered in 2002 (Gaudray G et al, 2001) and early publications on HBZ were exclusively based on usHBZ. The alternative transcript, sHBZ, was first reported in 2006 (Cavanagh MH et al, 2006). Since sHBZ is more abundantly expressed in infected cells (Yoshida M et al, 2008), current studies are mostly focused on sHBZ. The half-life of sHBZ is much longer than that of usHBZ (Yoshida M et al, 2008). HBZ expression has been correlated with HTLV-1 proviral load (Saito M et al, 2009). HTLV-1-infected rabbits were studied for kinetic analyses of the HBZ transcript which revealed that HBZ was detected at relatively low levels early after infection and its expression gradually increased and stabilized, whereas other viral genes were maintained continuously at a low level (Zhao T, 2016). Previous study demonstrated that the HBZ gene was expressed in all ATL cells and that HBZ gene knockdown inhibited the proliferation of HTLV-1-infected cells, indicating that HBZ may play a critical role in HTLV-1-mediated oncogenesis. HBZ is localized in the nucleus (Hivin P et al, 2005) and contains an activation domain (AD) at its N-terminus, a central domain (CD), and a basic leucine zipper (bZIP) domain

at the C-terminus. The N-terminus of HBZ has transactivating potential when fused with the DNA-binding domain of GAL4 and therefore it was named as the activation domain (Gaudray G et al, 2002). HBZ was first identified as a binding partner for the cAMP-response element binding protein-2 (CREB-2) by yeast two-hybrid screening (Hivin P et al, 2005).

Several studies have demonstrated that HBZ plays critical roles in ATL leukemogenesis and promotes cell proliferation (Matsuoka M and Green PL, 2009). Satou et al. reported that the HBZ mRNA enhances proliferation of T cells in culture and in transgenic mice (Satou et al, 2006). Also, the growth-promoting activity of HBZ was observed only in the cells expressing sHBZ and not in those cells expressing usHBZ (Yoshida M et al, 2008). Mitobe et al. reported that HBZ RNA increased the number of CD4<sup>+</sup> T cells and suppressed cell death by activating the transcription of the surviving gene, which inhibits apoptosis (Mitobe Y et al, 2015).

HBZ can interact with the bZIP domain of Jun, AP-1, and ATF/CREB- family of cellular transcription factors to suppress viral gene expression from the HTLV-1 5' LTR (Hilburn et al, 2011; Lairmore et al, 2011; Satou et al, 2006). HBZ is known to interact with cAMP response element-binding protein 2 (CREB-2) and suppresses Tax-mediated viral transcription through dimerization with CREB-2 preventing CREB-2 from binding to a Tax-responsive element in the 5'LTR of HTLV-1 (Gaudray G et al, 2002). HBZ also represses CREB-dependent transcription of cellular genes from a cellular cyclic AMP-responsive element (CRE) in the cyclin D1 promoter through its bZIP domain (Ma Y et al, 2013). Furthermore, HBZ inhibits Tax-dependent viral transcription by interacting with the KIX domain of p300/CBP through its LXXLL-like motifs located in the amino-terminus of HBZ and disrupting the Tax and p300/CBP complex (Clerc I et al, 2008). Additionally, HBZ can inhibit the NF $\kappa$ B signaling pathway by binding to the NF $\kappa$ B p65-subunit (Satou and Matsuoka, 2012; Zhao and Matsuoka, 2012). HBZ can also bind AP1-family

members (Activating Protein 1) including JunB, JunD and c-Jun to regulate both viral and cellular transcription (Zhao and Matsuoka, 2012; Kuhlman et al, 2007). HBZ has been shown to suppress AP-1 signaling pathway, which is mediated by c-Jun and JunB (Hivin P et al, 2005). However, HBZ can also form heterodimers with JunD and activate JunD-induced transcription and JunD-dependent cellular genes including human telomerase reverse transcriptase (hTERT) (Kuhlmann AS et al, 2007). This activation of telomerase by HBZ may contribute to the maintenance of and survival of leukemic cells (Zhao T, 2016). ATL, like other cancers, has up-regulated hTERT expression which may play a role in leukemogenesis (Zhao and Matsuoka, 2012; Kuhlman et al, 2007). Moreover, HBZ suppresses the canonical Wnt pathway and increases the expression of noncanonical Wnt5a which enhances the proliferation and migration of ATL cells which may be associated with the leukemogenesis of ATL (Ma G et al, 2013). Lastly, HBZ can up-regulate cellular proliferation genes while down-regulating apoptotic genes by interacting with Activating Transcription Factor 3 (ATF3), a member of the ATF/CREB family of transcription factors. ATF3 is constitutively expressed in ATL cell lines and all ATL cases. HBZ allows ATF3 to promote the proliferation of ATL cells by upregulating the expression of cell cycle regulatory genes (Hagiya et al, 2011).

HBZ plays a pro-survival role by suppressing the apoptosis of ATL cells. HBZ has been demonstrated to decrease cellular apoptosis by inhibiting the transcription of a proapoptotic gene Bim. HBZ interacts with FoxO3a, transcriptional activator of the Bim gene, and attenuates its DNA binding ability by epigenetic alterations and histone modifications in the Bim promoter region (Tanaka-Nakanishi A et al, 2014). Furthermore, Zhao et al. reported that HBZ protein selectively inhibits Tax-mediated classical NF- $\kappa$ B activation by inhibiting p65 DNA binding capacity and by promoting expression of the PDLIM2 E3 ubiquitin ligase, which results in p65

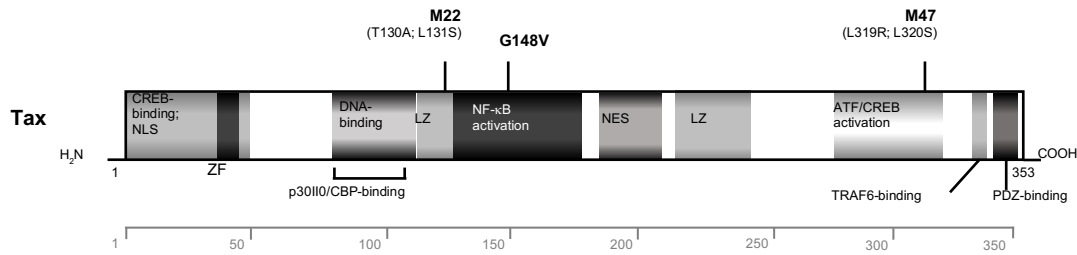
degradation. Thus, HBZ suppresses the onset of Tax-induced cellular senescence by down-regulating NF $\kappa$ B signaling (Zhao T et al, 2009). HBZ inhibits p65 acetylation and leads to repression of the classic NF $\kappa$ B pathway. Another study demonstrated that HBZ maintains viral latency by down-regulating Tax-mediated NF- $\kappa$ B activation and by inhibiting Rex-induced expression of viral proteins (Philip S et al, 2014). NF- $\kappa$ B signaling is a well-known mediator of host immunity. Hence it is possible that HBZ-mediated suppression of the classical NF $\kappa$ B pathway in ATL may facilitate escape from the host immune attack by decreasing the expression of genes associated with innate immunity and inflammatory responses (Saitoh Y et al, 2008). This role of HBZ also indicates towards a possible cooperative interplay between Tax and HBZ for the long-term development and maintenance of leukemic cells in ATL.

HBZ transgenic mice were found to have increased numbers of Foxp3<sup>+</sup>CD4<sup>+</sup> T cells. This population includes regulatory T cells (Tregs) which is a T-cell subset known to suppress effector T cells (Zhao T et al, 2011). It was seen that HBZ forms a ternary complex of HBZ/Smad3/p300 by interacting with Smad2/3, key components of the TGF- $\beta$  pathway, and leads to enhanced TGF- $\beta$ /Smad transcriptional responses in a p300-dependent manner. The enhancement of TGF- $\beta$  signaling by HBZ results in the overexpression of Foxp3 in naïve T cells. Thus, HBZ induces T cells to become Tregs by enhancing the TGF- $\beta$ /Smad pathway and upregulating Foxp3. As a mechanism, HBZ impairs the suppressive function of Treg cells by binding to Foxp3 and NFAT. HBZ interacts with Smad3 in a fashion similar to several other viruses that seem to employ a common strategy to nullify the TGF- $\beta$  signaling by having their viral proteins bind to Smad proteins (Zhao T, 2016). By impairing functions of conventional and regulatory T cells, HBZ critically contributes to the development of inflammation (Zhao T, 2016).



HBZ interacts with both viral and cellular factors and suppresses major HTLV-1 sense genes including Tax, promotes T-cell proliferation, suppresses cellular apoptosis and senescence, enhances viral infectivity, induces T-cell lymphoma and inflammation. Also, HBZ is the only detectable protein in patient isolates suggesting that HBZ expression plays an important role in HTLV-1- leukemogenesis (Zhao T, 2016; Yoshida et al, 2008; Satou et al, 2006). Taken together, these observations indicate that the HBZ gene is essential for cellular transformation and leukemogenesis of HTLV-1.

*Orf-IV (Tax)*: The tax gene encodes 353 amino acids long, 40-kD nuclear phosphoprotein. Tax is the potent transactivator of viral transcription. Tax has a nuclear localization signal at its amino terminus and hence it is predominantly present in the nucleus (Smith MR et al, 1992). However, a substantial portion of Tax is present also in the cytoplasm due to its leucine-rich (LR) nuclear export signal (NES) (Alefantis T et al, 2003). Tax has also been shown to be localized at extranuclear sites at the Microtubule-Organizing Complex (MTOC) (Semmes et al, 1996; Kfoury et al, 2012). Tax exists as a dimer and was originally discovered as a transactivator of viral RNA transcription from a promoter located at the 5'-LTR (Jin DY et al, 1997). Tax interacts with a number of cellular proteins and modulates a wide variety of cellular signaling pathways leading to transcriptional activation, proliferation and ultimately transformation. Tax also interacts with host transcription factors including CREB and recruits coactivators such as CREB binding protein (CBP) to the LTRs and regulates viral gene expression. Tax has pleiotropic functions and it regulates several cellular signaling pathways such as CREB, AP-1, NFAT and NF $\kappa$ B (Jeang KT, 2001; Azran I et al, 2004). Tax also dysregulates cell cycle control and inactivates tumor suppressors such as p53 and Rb (Jeang KT, 2001).



**Figure 3. Diagram of HTLV-1 Tax with its functional domains.** HTLV-1 Tax is a 353 aa long protein that contains a NLS: nuclear localization signal, NES: nuclear export signal, two (LZ) leucine zipper like regions and a dimerization domain. The mutations M22, G148V and M47 are also represented in the figure. It also contains p300/CBP binding domain and CREB binding domain in addition to binding domains for several other transcription factors. (Malu et al; 2019, submitted)

Several lines of evidence point towards the fact that Tax protein has the ability to initiate cellular transformation and immortalization. Grassman demonstrated that using a non-transforming Herpesvirus saimiri vector when the pX region of HTLV-1 was introduced into primary T cells, it resulted in the constitutive high-level expression of functional IL-2 receptors. Unlike the normal activated T cells, these pX-expressing T-lymphocytes could be maintained in continuous long-term culture in the presence of exogenous IL-2 (Grassman R et al, 1989), suggesting that the HTLV-I pX gene products may deregulate the normal transient nature of the T-cell growth response. Moreover, in the transgenic mice expressing the tax gene, multiple mesenchymal tumors and neurofibromas were seen (Hinrichs S et al, 1987). When Tax is co-expressed with Ras, it induces the formation of cellular foci and these cells when injected into nude mice led to tumor formation (Pozzatti et al, 1990). Similarly, when tax gene was stably expressed in a rat fibroblast cell line (Rat-1), it resulted in cellular transformation and demonstrated contact inhibition and anchorage independence in these cells (Tanaka A et al, 1990).

Tax is highly immunogenic and is one of the principal targets of cytotoxic T cells (CD8<sup>+</sup> T lymphocytes). Hence, in order to evade the host's adaptive immunity HTLV-1 uses multiple mechanisms to downregulate viral antigens, including Tax, and enter a state of latency. The viral regulatory proteins HTLV-1 basic leucine zipper factor (HBZ), p30<sup>II</sup> and Rex exert negative effects on Tax expression and help in immune evasion (Nicot C et al, 2004; Anupam R et al, 2013). The HTLV-1 Tax protein is required for oncogenic transformation of infected cells (Akagi T et al, 1995); however, tax transcripts are detected in only about 40% of all ATLs. Further studies of HTLV-1 proviruses and tax transcripts in ATL cells revealed three ways in which cells can silence Tax expression: genetic changes are seen in ~10% of ATL cases (nonsense mutations, insertions and deletions in tax) (Furukawa Y et al, 2001; Takeda S et al, 2004); in ~15% of ATL cases DNA methylation of the provirus leads to silencing of viral transcription (Takeda S et al, 2004; Koiwa T et al, 2002; Taniguchi Y et al, 2005); and lastly, in ~27% of ATL cases deletion of 5' LTR of the provirus is observed (Tamiya S et al, 1996). While it seems likely that Tax may play a crucial role in the development of ATL, Tax may not be required for the maintenance of this transformed phenotype. Later it was accepted that Tax is needed in early stages of virus infection to initiate transformation, but it is not required later for maintaining the transformed phenotype of ATL cells (Tamiya S et al, 1996).

Activation of viral transcription by Tax: CREB/ATF transcription factors are involved in cell growth, survival and apoptosis by regulating CRE- directed gene transcription in response to growth factor signals or stress (Taylor G, 2007). The U3 region of the HTLV-1 LTR contains a region known as Tax responsive element 1 (TxRE-1 or TRE1) composed of three discontinuous 21 base pair repeats (Seiki et al, 1983). The TRE element contains an octamer motif that is flanked by a G-rich sequence at the 5' end and C-rich sequence stretch at the 3' end. This octamer shares

homology with the consensus cAMP-responsive elements (CRE) 5'-TGACGTCA-3' (Yin MJ and Gaynor RB, 1996). Tax can form stable ternary complexes with cellular transcription factors CREB and ATF-1 that contain a basic leucine zipper domain (b-ZIP). In these ternary complexes, CREB/ATF-1 bind the CRE of the 21-bp repeats and Tax binds the b-ZIP domains of CREB/ATF-1 (Adya N et al, 1994; Yin M et al, 1995; Baranger AM et al, 1995; Perini G et al, 1995) and makes contacts with the DNA minor groove of the G/C-rich sequences that flank the CRE. This allows for high DNA sequence specificity of LTR transactivation by Tax (Lundblad JR et al, 1998). Thus, Tax increases CREB/ATF dimerization and their affinity for viral CREs by stabilizing the ternary complex through direct contact with the GC-rich flanking sequences (Taylor G, 2007; Boxus M et al, 2008; Yin MJ and Gaynor RB, 1996; Lundblad JR et al, 1998). Furthermore, Tax recruits the co-activators CBP/p300 and P/CAF and facilitates transcription initiation, binds to CREB-binding protein (CBP), and forms a bridge between CREB and CBP. This ternary complex consisting of TAX/CREB/TRE is stabilized through Tax binding and the recruitment of the co-activators p300/CBP and P/CAF resulting in histone acetylation, opening of the nucleosome and transcriptional activation (Harrod R et al, 1998; Harrod R et al, 2000; Boxus M et al, 2008) allowing positive regulation of viral promoter by Tax. A number of cellular genes contain a consensus CRE element in their promoters which are activated by elevation in CAMP levels. The elevated CAMP activates PKA which phosphorylates CREB and in turn, CREB binds to CBP/p300. It is possible that Tax can activate the CRE located in cellular promoters in addition to the viral CRE activation. There are some data that demonstrate that Tax uses the CREB/ATF factors to repress the expression of certain genes including cyclin A, p53 and c-myc (Boxus M et al, 2008). This activation of CREs by tax may play a role in initiation of oncogenic process by dysregulating the cell cycle control and growth control.

Tax-mediated NF $\kappa$ B activation: The NF $\kappa$ B family of transcription factors regulate the expression of over 100 target genes and is known to regulate diverse biological processes and the expression of genes involved in immune responses, development, cell proliferation, apoptosis and survival (Ghosh S et al, 1998). Deregulation of NF $\kappa$ B has been associated with several human diseases, specially cancers (Xiao F and Fu J, 2011). The NF $\kappa$ B pathway is a lucrative target to viral pathogens because the activation of NF $\kappa$ B is a rapid immediate early (IE) event that occurs within minutes following exposure to a stimulant and does not require any de novo protein synthesis and yet it results in a strong transcriptional activation of many cellular genes as well as early viral genes (Hiscott J et al, 2001). The NF $\kappa$ B family consists of Rel family members which function as homo- and hetero-dimers and share a highly conserved 300-amino acid-long N-terminal Rel homology domain (RHD) which is also responsible for their dimerization, nuclear translocation, DNA binding and interaction with the inhibitors of NF $\kappa$ B (I $\kappa$ Bs). Rel family includes five closely related DNA binding proteins: RelA (p65), RelB, c-Rel, NF $\kappa$ B1/p50 and NF $\kappa$ B2/p52, which regulate transcription of genes containing  $\kappa$ B motifs in their promoters (Xiao G et al, 2006). The p50 and p52 proteins are generated from their precursors p105 and p100 respectively, by proteasome-mediated degradation. In resting cells, Rel proteins are sequestered in the cytoplasm through their interactions with the inhibitory proteins I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ , as well as the precursor proteins p105 and p100. Upon stimulation, the I $\kappa$ B kinase (IKK) complex, which is composed of 2 catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and the scaffolding protein, NF $\kappa$ B essential modulator (NEMO, also known as IKK $\gamma$ ) gets activated. The IKK complex phosphorylates specific serine residues on the I $\kappa$ B proteins leading to their poly-ubiquitination and proteasome-dependent degradation or processing (Baldwin AS Jr., 2001; Hayden MS and Ghosh S, 2004). These released NF $\kappa$ B then translocates to the nucleus and regulates the expression of target genes.

Moreover, the gene encoding I $\kappa$ B $\alpha$  is also under the control of NF $\kappa$ B and thus provides a negative feedback loop to control the NF $\kappa$ B activation of its downstream cellular gene targets (Sun SC and Ballard DW, 1999).

Persistent NF $\kappa$ B activation by HTLV-1 Tax is the key event for T-cell transformation and the development of ATL (Qu Z et al, 2011; Zhang H et al, 2016). Despite the fact that the HTLV-1 proviral genome is integrated and NF $\kappa$ B is constitutively activated in ATL cells, Tax is expressed in only about 40% ATL cells, however HBZ is consistently expressed in all ATL cells (Zhao T, 2016). This suggests the possible contribution of additional factors in order to sustain the persistent activation of NF $\kappa$ B even in the absence of Tax in ATL cells (Matsuoka and Jeang, 2007). The regulation of NF $\kappa$ B pathway by HTLV-1 Tax is discussed in further detail in a separate section of this dissertation.

Cell-cycle deregulation and DNA damage by Tax: HTLV-1 Tax has been implicated to play a crucial role in cell cycle control. By disrupting cell cycle check points, Tax prevents apoptosis/cell death and promotes the proliferation of infected cells conferring an obvious advantage to the virus infected cell. The interaction of Tax with cell cycle components often results in dysregulation leading to cellular abnormalities including aneuploidy and immortalization of T-cells, all of which play key roles in oncogenesis (Curren R et al, 2012). Cell cycle progression is tightly regulated by cyclins and cyclin-dependent kinases (CDK). Passage from G1 phase and G1/S transition is carried out by Cyclin D-CDK4/6 and Cyclin E-CDK2 complexes respectively. Tax has been shown to activate G1/S entry. Specifically, Tax can disrupt the G1/S transition by its direct interaction with CDK4 and CDK6 via its amino terminal domain. Through these interactions, Tax stabilizes the cyclin D2/CDK4 complex and enhances its kinase activity, leading to hyperphosphorylation of retinoblastoma protein (Rb) (Ohtani K et al, 2000; Jeang KT et al, 2004; Schmitt I O et al, 1998).

G1/S progression can be inhibited by CDK inhibitors (CDKI) such as p15INK4b, p16INK4a, p18INK4c and p19INK4d that bind to CDKs and make them unavailable for cyclin binding. Tax associates with p15INK4b and p16INK4a and prevents it from binding to CDK4 and CDK6 (Low KG et al, 1997). Finally, Tax has also been shown to directly bind to the hypo-phosphorylated form of Rb and targets it for proteasomal degradation (Kehn K et al, 2005).

Another cell cycle checkpoint that the cell passes is while going from G1 to S phase and is regulated by the tumor suppressor p53. p53 is mutated in approximately half of all human cancers, but interestingly in ATL p53 is often wild-type (Mulloy et al, 1998; Pise-Masison and Brady, 2005; Zane et al, 2012). In case of DNA damage, p53 is activated by ATM/ATR DNA damage recognition pathways and signals for repair (Zhou and Elledge, 2000). Tax disables the p53-mediated G1/S checkpoint, allowing cell cycle progression to occur even in the presence of DNA damage (Haoudi and Semmes, 2003). Tax activates expression of the p53 target gene, p21waf1 in a p53 independent manner (De La Fuente et al, 2000). The ability of Tax to induce p21waf1 expression in HTLV-1-immortalized cells is associated with resistance to apoptosis (Kawata et al, 2003). Tax may also inhibit the G1/S checkpoint by inactivating p53 (Mulloy et al, 1998; Pise-Masison et al, 1998). Tax neither binds to p53 nor represses its transcription. However, it was shown that in the presence of Tax expression there is nuclear stabilization of p53 (Cereseto et al, 1996). There are two major theories to explain the inactivation of p53 by Tax. In some studies, it was shown that Tax and p53 compete for binding to the ubiquitous coactivator CBP/p300, thereby decreasing the ability of p53 to activate target gene expression (Suzuki et al, 1999b; Van et al, 2001). Another mechanism of p53 inactivation is through NFκB signaling pathway (Pise-Masison et al, 2000). Tax was shown to facilitate the formation of functionally inactive complexes containing p65<sup>RelA</sup> and p53, and this interaction requires p53-phosphorylation on serine 15. This

Ser-15 site was previously shown to be preferentially phosphorylated in Tax-expressing cells (Jeong et al, 2004). Also, through repressing p53-dependent transcription, the p53-dependent gene Bax is also repressed and inhibits the induction of apoptosis (Satou and Matsuoka, 2012; Kfoury et al, 2012). By preventing several p53-dependent apoptotic pathways, cells can continue to proliferate and may lead to a defective DNA damage response seen in Tax-expressing cells. Anaphase promoting complex (APC) is a E3-ubiquitin ligase that functions to direct ubiquitin-mediated degradation of anaphase inhibitors. A complex between the APC and its accessory factor Cdc20p, known as APC<sup>cdc20p</sup>, ubiquitinates cyclin B1 as well as the Pds1p/securin protein and targets them for degradation. Liu et al. demonstrated that Tax directly binds to and prematurely activates APC<sup>cdc20p</sup>, leading to premature degradation of cyclin B and causing delayed cell cycle progression. This also leads to an improper sister chromatid separation and contributes to aneuploidy. This interaction between Tax and APC-related complexes may explain the mitotic defects observed in Tax-expressing cells (Liu et al, 2003; 2005). Several cellular proteins including the mitotic arrest defective (MAD) proteins 1 and 2 are responsible for securing this checkpoint. MAD1 was identified as a binding partner of Tax, and it was observed that Tax binds to hMAD1 and causes prominent cytokinesis defects in the form of multinucleation Tax-expressing cells (Jin et al, 1998).

#### *HTLV-1 associated Pathogenesis*

ATL is one the most aggressive disease associated with HTLV-1 infection. In addition to it there are other HTLV-1 associated conditions including HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP), infective dermatitis associated with HTLV-1 (IDH) and an inflammatory eye condition called HTLV-1 associated uveitis (HAU). HAM/TSP can manifest in



about 3-5% of HTLV-1 infected individuals. It commonly affects women with an average age of 40 years at disease onset. HAM/TSP is a neurological condition manifested due to lesions in central nervous system (CNS) as a result of infiltration of HTLV-1 infected CD4+, CD8+ T cells and proinflammatory cytokines and chemokines into the CNS. It involves spasticity of lower limbs, bowel and bladder disturbances and a slow and steady progression over several years. While ATL, HAM/TSP, and ID are the major diseases associated with HTLV-1- infection, eyes, skin, joints, and bone are also affected by HTLV-1. Some HTLV-1-positive individuals develop Sézary syndrome, a cutaneous T-cell lymphoma (Green et al, 2001). Autoimmune disorders, like Sjögren syndrome may be associated with chronic lymphocytic infiltration in both salivary and lacrimal glands, (Goncalves et al, 2008).

TSP/HAM: This is a chronic meningomyelitis of the gray and white matter in the spinal cord with perivascular demyelination and axonal degeneration that usually begins in adulthood. TSP/HAM is characterized by weakness and spasticity of the extremities, mild peripheral sensory loss, and hyperreflexia (Cooper SA et al. 2009). The majority of TSP/HAM patients are seropositive for anti-HTLV-1 antibodies (Osame et al, 1987) and polyclonal integration of proviral DNA is common. There is presence of morphologically atypical lymphocytes in the peripheral blood. There have been cases where HAM/TSP developed within months of infection by contaminated blood products; this is significantly different from ATL, which usually develops after an incubation period of decades. In a few cases however, ATL and TSP/HAM have been known to occur in the same patient. HTLV-1 DNA can be found in blood and CSF lymphocytes (Yoshida et al, 1987). High levels of HTLV-1 specific cytotoxic T-lymphocytes (CTLs) have been detected in TSP/HAM patients that may contribute to the neurological damage (Johnson JM et al, 2001). It has been suggested that the central nervous system (CNS) damage may be due to the

recognition of infected cells which are then lysed by the host's immune system. HTLV-1 can infect neuronal cells in vitro and in vivo (Yoshida et al, 1987). An alternate way of damage may be indirectly due to autoimmune or cytokine-mediated mechanisms since there is a marked increase in levels of TNF- $\alpha$ , GM-CSF, IFN- $\gamma$ , and IL-1 in TSP/HAM patients unlike those in asymptomatic carriers (Watanabe et al, 1995). There is no curative treatment for TSP/HAM and the prognosis depends on the individual immune response and proviral loads. Corticosteroids, IFN- $\alpha$ , and IFN- $\beta$ 1a have shown limited results (Nakagawa MK et al, 1996).

Infective Dermatitis associated with HTLV-1 (ID): HTLV-1 is often associated with dermatological lesions. ID was the first pediatric syndrome associated with HTLV-1 infection. ID is a severe recurrent childhood eczema with the first symptoms manifesting at approximately 18 to 24 months. Children with IDH are vertically infected through breast milk of HTLV-1 seropositive mothers (De Oliveira et al, 2010). It is characterized by a severe and relapsing eczematous disease with lesions involving the scalp, neck, external ear, axillae, and groin (La Grenade L et al, 1990). ID is a very important manifestation because cutaneous involvement in an asymptomatic carrier has been considered an indicator of future ATL or HAM/TSP development (Goncalves DU et al, 2010). Follicular papules and pustules on affected areas is seen. ID patients yield positive cultures of *Staphylococcus aureus* and beta-hemolytic *Streptococcus* for nostrils or skin (La Grenade L et al, 1990). An adequate response to systemic antibiotics and topical steroids is usually obtained, although relapse is common (La Grenade L et al, 1990). IDH usually disappears by adolescence; however, the patient may develop ATL or HAM/TSP in adulthood (De Oliveira et al, 2010).

HTLV-1-Associated Uveitis (HAU): HAU is characterized by a granulomatous or non-granulomatous reaction accompanied by vitreous opacities and retinal vasculitis in one or both

eyes (Mochizuki M et al, 1992). It can occur as the only HTLV-1 manifestation or can be associated with HAM/TSP. HAU target population are either male or female HTLV-1-infected middle-aged adults. “Flying flies” and acute or sub-acute visual blurring characterized by ‘floaters’ and foggy vision in the eye are characteristic symptoms associated with HAU (Mochizuki M et al, 1992; Goncalves DU et al, 2010). Uveitis was more frequent among those with an earlier onset of HAM/TSP and in patients with severe motor disability (Goncalves DU et al, 2010). The immune system of patients with HAU has abnormalities similar to those seen for patients with HAM/TSP including high proviral load, overproduction of proinflammatory cytokines, and the presence of HTLV-1 provirus in the spinal fluid. HAU can be either an independent inflammatory disease associated with HTLV-1 or, perhaps, a predictor of HAM/TSP. HAU in the Caribbean and Brazil areas is observed to manifest the corneal disease which was not described for Japanese patients (Mochizuki M et al, 1992). The treatment of uveitis is based on topical and systemic corticosteroids. Usually the response to therapy is satisfactory and the patients are able to get their vision back to normal, although recurrence is also common. In order to prevent ocular complications such as corneal ulcers lubricating drops are widely used (Goncalves DU et al, 2010).

### *Characteristics of ATL*

HTLV-1 infection sometimes results in development of an aggressive non-Hodgkin’s peripheral T-cell malignancy- ATL. Most of the HTLV-1 infected individuals remain asymptomatic throughout their lifetime except about 5% that go on to develop ATL after a long latency period of 40-50 years. The disease is associated with diverse clinical features including lymphadenopathy, hepatosplenomegaly, skin lesions, blood and bone marrow involvement, hypercalcemia and lytic bone lesions. Shimoyama classification (Lymphoma Study Group classification) that takes into account lymphocytosis, tumor syndrome, hypercalcemia, and lactate

dehydrogenase (LDH) values recognizes four ATL subtypes: acute and lymphoma aggressive forms, chronic and smoldering indolent forms (Shimoyama M, 1991). Wattel E et al have described the borderline state between healthy carriers of HTLV-1 and ATL patients as pre-ATL (Wattel E et al, 1995). Pre-ATL is an asymptomatic phase and is characterized by the presence of abnormal peripheral blood lymphocytes with characteristic ATL-cell morphology. From the pre-ATL phase, a patient can either go back to normal or progress to one of the subtypes of ATL. The clinical features of ATL are dependent on the disease type (Shimoyama M, 1991). **Acute ATL** (so-called prototypic ATL) comprises 60% of all ATL cases and has a leukemic presentation. Acute ATL presents with lymphadenopathy, hepatosplenomegaly, lytic bone lesions, skin lesions and systemic multi-organ involvement. Hypercalcemia is frequently observed along with diverse opportunistic infections due to general immunosuppression (Takatsuki K et al, 1985; Bazarbachi A et al, 2011). 20% of all ATL cases are referred to as **lymphoma type ATL** and have lymph node enlargement with < 1% of leukemic cells circulating in the peripheral blood. The lymphoma type of ATL is considered to be a form of T-cell-type non-Hodgkin's lymphoma. These patients may also present with hepatosplenomegaly, lytic bone lesions, skin lesions, gastrointestinal, and lung infiltration which are usually less frequent than in acute-type ATL (Bazarbachi A et al, 2011). The **smoldering type ATL** presents infiltration of skin or lung with no other visceral involvement, a normal lymphocyte count, and about 1-5% abnormal lymphocytes in the peripheral blood (Tsukasaki K et al, 2013; Bazarbachi A et al, 2011; Shimoyama M, 1991). **Chronic-type ATL** is associated with high lymphocyte count and chronic peripheral lymphocytosis for several years associated with skin and lung involvement, lymphadenopathy, and hepatosplenomegaly. Chronic ATL however does not have any associated hypercalcemia or infiltration of the CNS, gastrointestinal tract, or bones. Lactate dehydrogenase levels are normal or only slightly increased

(Tsukasaki K et al, 2013; Bazarbachi A et al, 2011; Shimoyama M, 1991). Chronic-type ATL is further sub-grouped into favorable and unfavorable. Unfavorable subtype is defined by low serum concentration of albumin, high lactate dehydrogenase, and a high expression of Ki-67 antigen (Shirono K et al, 1994; Yamada Y et al, 2001).

ATL patient serum contains antibodies to HTLV-1 and in most cases, the HTLV-1 provirus is clonally integrated in the CD4<sup>+</sup> CD25<sup>+</sup> -activated T lymphocytes, which have the characteristic flower shaped nuclei. Although the exact mechanism is not known but this appears to be the first step in the multistep HTLV-1 related oncogenesis (Franchini G, 1995). ATL cells express the IL2 alpha receptor (IL2-R, CD25) and show a constitutive activation of NF-kappa B pathway. Structural and numeric karyotypic abnormalities are seen more frequently in aggressive ATL cases than in indolent ATL (Kamada N et al, 1992). The most frequent aberrations included gains at chromosomes 14q, 7q, and 3p and losses at chromosomes 6q and 13q. (Tsukasaki et al, 2001). In ATL cases, the gene deletions tend to cluster around cyclin dependent kinase inhibitors and cell cycle regulatory genes such as p15 (INK4A), p16 (INK4B), p18 (INK4C), p19 (INK4D), p21 (WAF1), p27 (KIP1), as well as p53 and retinoblastoma (Rb) (Hatta and Knoeffler, 2002). The p53 tumor suppressor is mutated in nearly half of all cancers, but p53 mutations are rarely observed in ATL patient samples (Hatta and Knoeffler, 2002). However, it was observed that the p53 gene was mutated in 10% to 50% of aggressive type ATL cases, whereas its frequency was much lower in indolent-type ATL (Cesarman E et al, 1992). These studies indicate that mutations in the cell cycle-related genes are more likely to be associated with more severe stages of ATL than with earlier clinical stages of this malignancy.

Furthermore, ATL cells have an intrinsic resistance to various chemotherapies. All these factors taken together result in very poor prognosis for ATL disease, and a median survival of 6.2,

10.2, and 24.3 months for acute, lymphoma, and chronic subtypes, respectively (Shimoyama M, 1991).

#### *Current Therapies and Treatment of ATL*

ATL is diagnosed by testing the sera for anti-HTLV-1 positivity and checking the presence of a mature T-cell malignancy. To confirm the diagnoses, identification of monoclonal integration of HTLV-1 proviral DNA in tumor cells is done by Southern blot analysis ATL (Shimoyama M, 1991). The treatment strategy for ATL is universally based on certain factors including clinical subtypes, prognostic factors and response to initial therapy (Utsunomia A et al, 2015). Patients with aggressive ATL (acute and lymphoma types and chronic type with poor prognostic factors) are recommended intensive chemotherapy regimens such as VCAP-AMP-VECP (vincristine, cyclophosphamide, doxorubicin, and prednisone; doxorubicin, ranimustine, and prednisone; vindesine, etoposide, carboplatin, and prednisone) or AZT/IFN- $\alpha$  combination therapy (zidovudine/interferon  $\alpha$ ), except for in cases with lymphoma. In cases of indolent ATL (smoldering type and chronic type without poor prognostic factors), either waiting with careful observation or AZT/IFN- $\alpha$  combination therapy is recommended until disease progression is seen. In both United States and Europe, leukemic-type ATL is treated using AZT/ IFN- $\alpha$  antiviral therapy. However, for lymphoma-type ATL, the overall survival with AZT/ IFN- $\alpha$  alone is very short hence chemotherapy is the first-line therapy in such cases (Bazarbachi A et al, 2010). A small-scale study has also reported the efficacy of the combination of AZT/IFN- $\alpha$  and arsenic trioxide in chronic ATL patients (Kchour G et al, 2009). Moreover, the addition of arsenic trioxide was shown to induce Tax degradation in vitro (Dassouki Z et al, 2015). Due to mutations in multidrug resistance (MDR) genes and tumor suppressor genes like p53, there is approximately 10% reduction in long term treatment success making ATL very resistant to therapy (Taylor and

Matsuoka, 2005; Matsuoka, 2003; Bazarbachi et al, 2011). More recently other strategies such as hematopoietic stem cell transplantation for relatively young patients with aggressive ATL are also being applied (Utsunomia A et al, 2015).

### *Animal Models of HTLV-1 and ATL*

Researchers started to develop animal models to understand the biology of HTLV-1 infection shortly after the discovery of HTLV-1. Animal models are of great use as they can help to develop vaccines, study the disease progression and test new therapies. HTLV-1 can infect animals, such as rabbits, rats, and monkeys (Akagi et al, 1985; Nakamura et al, 1987). HTLV-1 infected rats were shown to develop a chronic progressive myeloneuropathy which was similar to HAM (Kasai et al, 1999). Over years several animal models for ATLL have been described and developed in rats and rabbits (Ohashi et al, 1999; Simpson et al, 1996). However, it was seen that although leukemic cells can be transplanted in these models, leukemia of rat or rabbit origin does not develop in these animals. Transgenic animals are being used more recently to examine the effect of single or multiple viral genes, particularly the viral transactivator Tax, on HTLV-1 associated leukemogenesis. Tax has been shown to play an important role in the induction of tumors in transgenic mice (Hinrichs et al, 1987; Grossman et al, 1995; Yamada et al, 1995).

**Rabbit Models:** It has not been easy to establish a clinical HTLV-1 disease model in rabbits. Although, infectivity in rabbits was demonstrated early in the mid-1980s using intravenous inoculations of an ATL patient T-cell leukemia cell line known as the MT-2 cell line (Akagi et al, 1985), and a rabbit lymphocyte cell line derived from cocultivation of rabbit lymphocytes with MT-2 cells, called the Ra-1 cell line (Miyoshi et al, 1985). This initial study also demonstrated that HTLV-1 was transferable via blood transfusions in rabbits (Miyoshi et al, 1984; 1985) and that cell-free plasma was not capable of transmitting the virus to rabbits (Kotani S et al, 1986). In

most of these studies, rabbit infection has been like the asymptomatic infection of humans. Some groups have tried to reproduce an 'ATL-like disease' via intraperitoneal or intravenous injection of HTLV-1-transformed cells; however, this required a huge number of cells and death occurred within the first few weeks of inoculation (Lairmore MD et al, 2005). The early studies in rabbits helped verify routes of transmission of HTLV-1 (e.g. blood, semen, milk) (Kotani et al, 1986). Infectious molecular clones of HTLV-1 were first developed in the mid-1990s (Kimata et al, 1994). The molecular clones were used to immortalize human PBMCs to create the ACH cell line, which was then used to infect rabbits to demonstrate that the lethally irradiated ACH cell line could successfully establish infection in the PBMCs of rabbits (Collins et al, 1996). Later, mutant ACH molecular clones disrupting some HTLV-1 accessory genes within the ORF, including, p12<sup>I</sup>, p13<sup>II</sup>, and p30<sup>II</sup>, were generated and inoculated into rabbits to analyze their importance during in vivo infection (Silverman et al, 2004). The regulatory gene, Rex, and accessory genes, p12 and p30<sup>II</sup>, have all been demonstrated as dispensable for viral immortalization in vitro (Lairmore MD et al, 2005).

Rat Models: Rats as experimental models for HTLV-1 infection were first established in 1991 (Suga et al, 1991). It was established that there were considerable differences in the response of various rat strains to HTLV-1 infection (Lairmore MD et al, 2005). The HTLV-1-infected Wistar-King-Aptekman (WKA) strain of rats were concluded to produce an antibody response to HTLV-1 and was established as a model for TSP/HAM (Yoshiki T, 1995). Moreover, rat models have been proven to be a valuable tool for studying mother-to-child transmission of HTLV-1 by vertical transmission as well as intrauterine transmission (Lairmore MD et al, 2005). Immunodeficient rats were inoculated with some specific HTLV-1-immortalized cell lines to develop an 'ATL-like lymphoproliferative disease' model (Ohashi T et al, 1999).



Non-human Primate Models: Miyoshi et al in 1984 first reported that HTLV-1 infection is possible in non-human primates by intravenous inoculation of HTLV-1 producing Ra-1 cell line into Japanese monkeys which resulted in antibody production and seroconversion (Miyoshi et al, 1984; 1985). Squirrel monkeys were also inoculated with a HTLV-1 producing cell line and although no signs of ATL development were observed until two years after inoculation, peripheral lymphocytes, spleen, and lymph nodes were verified as major reservoirs for HTLV-1 virus during the early phase of infection (Kazanji M et al, 2000). It was later reported that, HTLV-1 infection in squirrel monkeys begins through reverse transcription of the virus genome, followed by clonal expansion of infected cells, a process similar to that seen in humans (Mortreux et al, 2001). HTLV-1 infection in macaques can lead to human-like manifestations of disease including arthritis, uveitis, and polymyositis (Beilke MA et al, 1996) and various combinations of rash, diarrhea, lymphadenopathy, and lymphopenia (McGinn TM et al, 2002). Many studies used cynomolgus and squirrel monkeys, Japanese macaques for testing vaccines and passive immunization (Lairmore MD et al, 2005).

Mouse models: Similar to rabbit and rat models, HTLV-1 doesn't efficiently infect murine cells either. Hence in order to maintain persistent infection, mouse models were manipulated by using intra-peritoneal inoculation. The inoculation of immunocompetent mice is similar to in vivo human infection in several ways. A broad spectrum of infected cell types were seen in infected mice including CD4+, CD8+, B cells, and granulocyte fractions, similar to those observed in HTLV-1-infected individuals. Furthermore, infected mice present polyclonal proliferation of spleen infected cells and lymphatic tissues that act as a reservoir for infected and proliferating cell clones. Apart from one report of tumor induction, immunocompetent HTLV-1 carrier mice have

not provided any significant evidence of the in vivo spread of infection and any apparent disease after infection (Panfil AR et al, 2013).

In 1992 Ishihara et al successfully used severe combined immunodeficiency (SCID) mice to model the proliferative and tumorigenic potential of ATL cells in mice. They found that some of the mice developed tumors at or near the injection site, which were positive for detection of viral p19 and also proviral DNA. Repression of SCID mouse NK cells using an anti-murine IL2 receptor beta chain monoclonal antibody followed by injection with ATL patient cells resulted in tumor and leukemia development in about 5–7 weeks after inoculation. Moreover, they identified the proliferating leukemic cells in the mouse as deriving from the original inoculating ATL cell clone. They also saw the infiltration of ATL cells into various mouse organs which was similar to what is observed in ATL patients (Ishihara S et al, 1992). Ohsugi et al demonstrated successful tumorigenesis from MT-2 cell engraftment in SCID mice with normal NK cell function as opposed to the previous experiments that utilized reduced NK cell function to establish tumors (Ohsugi T et al, 2004).

Tax transgenic mice: Nerenberg et al in 1987, first described HTLV-1 promoter (LTR)-Tax transgenic mice (Nerenberg M et al, 1987). In these mice, Tax protein expression efficiently induced mesenchymal tumors and had tissue-specific expression patterns. Iwakura Y et al developed Tax Tg mice that has Tax expression in the brain, salivary gland, spleen, thymus, skin, muscle, and mammary gland tissues (Iwakura Y et al, 1995). It was observed that these Tg mice had a higher incidence of mesenchymal tumors and adenocarcinomas with activated c-Fos and c-Jun expression in the tumors. In other Tg mice, Tax expression was restricted to the lymphoid cell compartment by the use of alternative promoters. Tax was over expressed under the human granzyme B promoter to target expression to the mature T-lymphocyte compartment which

resulted in large granular lymphocytic leukemia/lymphomas in mice. These Tax Tg mice also spontaneously developed hypercalcemia, high frequency osteolytic bone metastases, and enhanced osteoclast activity, symptoms frequently associated with ATL development (Grossman WJ et al, 1995). Under the control of the Lck proximal promoter, Tax expression is restricted to developing thymocytes and this model was able to demonstrate lymphomas characterized by constitutive NF $\kappa$ B activation and increased cytokine and growth factor expression (Hasegawa H et al, 2006). These studies in various transgenic mice confirm HTLV-1 as a transforming virus and Tax as its oncogenic protein.

### **1.3 Role of NF $\kappa$ B inflammatory signaling in HTLV-1 infected cells**

HTLV-1 is an animal oncoretroviruses that does not carry host-derived oncogene, neither does it activate a cellular oncogene through proviral integration. HTLV-1 encodes a regulatory protein Tax which is the primary oncogenic mediator. Tax does not bind DNA directly, instead it induces cellular target genes indirectly by its pleiotropic control on host cellular pathways (Smith MR and Greene WC, 1991). Among those host machineries, NF $\kappa$ B signaling plays a pivotal role in Tax-mediated transformation and ATL leukemogenesis (Ballard DW et al, 1988).

In normal T-cells NF $\kappa$ B activation is tightly regulated and occurs transiently in response to immune stimuli, playing an important role in T-cell proliferation and survival. Moreover, under normal conditions, following stimulation by an antigen, T-cells predominantly stimulate the canonical NF $\kappa$ B pathway ensuring a rapid but short-lived nuclear expression of NF $\kappa$ B responsive genes (Ballard et al, 1988; Xiao et al, 2001). T cell receptor (TCR and its proximal signaling molecules are downregulated shortly after antigen stimulation, preventing persistent signaling through the cell surface receptor (Jang and Gu, 2003). There is also a negative feedback mechanism through which the activated NF $\kappa$ B induces increased expression and synthesis of

inhibitory I $\kappa$ B $\alpha$  molecules that curb the further NF $\kappa$ B pathway activation (Sun et al, 1993). However. In case of HTLV-1 infection, the virus induces persistent activation of NF $\kappa$ B, leading to deregulation of an array of NF $\kappa$ B responsive antiapoptotic, proliferation, and cell cycle progression genes (Fochi S et al, 2018). Constitutively high NF- $\kappa$ B activity has typically been observed in human hematopoietic cancer cells, including adult T-cell leukemia (ATL), Hodgkin lymphoma, and multiple myeloma cells.

### *The NF $\kappa$ B family*

Nuclear factor-kappa B (NF $\kappa$ B) is a family of transcription factors involved in regulation of immune response, cell proliferation, survival and development. Several human diseases, particularly cancers, have been associated with deregulated NF $\kappa$ B (Xiao G and Fu J, 2011). The NF $\kappa$ B family consists of five DNA binding proteins that share a 300 amino acid long N-terminal Rel homology domain (RHD) and contain  $\kappa$ B motifs in their promoters: RelA (p65), RelB, c-Rel, NF $\kappa$ B1/p50 and NF $\kappa$ B2/p52 (Ghosh and Hayden, 2008). The RHD allows nuclear translocation, DNA binding, interaction with the inhibitors of NF- $\kappa$ B (I $\kappa$ Bs), formation of homo- and heterodimers in these proteins. RelA, RelB and c-Rel are directly synthesized in their mature form and contain a C-terminal transactivating domain (TAD) whereas p50 and p52 are generated from large precursor proteins, p105 and p100, respectively. p100 and p105 can also function as NF $\kappa$ B inhibitors since they contain I $\kappa$ B-like sequences in their C-terminal (Rice NR et al, 1992).

In resting cells, Rel proteins are sequestered in the cytoplasm through their interactions with the inhibitory proteins I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , and I $\kappa$ B $\epsilon$ , as well as the precursor proteins p105 and p100. Upon stimulation, the I $\kappa$ B kinase (IKK) complex, which is composed of 2 catalytic subunits, IKK $\alpha$  and IKK $\beta$ , and the scaffolding protein, NF- $\kappa$ B essential modulator (NEMO, also known as IKK $\gamma$ )

gets activated. The IKK complex phosphorylates specific serine residues on the I $\kappa$ B proteins leading to their poly-ubiquitination and proteasome-dependent degradation or processing (Baldwin AS Jr, 2001; Hayden MS and Ghosh S, 2004). These released NF- $\kappa$ B then translocates to the nucleus and regulates the expression of target genes.

#### *Canonical and Non-Canonical NF $\kappa$ B pathways*

The NF $\kappa$ B signaling pathways can be activated by variety of signals through two distinct pathways: the canonical and noncanonical pathways. Generally, the canonical pathway regulates inflammation and cell survival, whereas the noncanonical pathway regulates lymphoid organogenesis and B-cell survival and maturation and bone metabolism (Sun SC, 2011). The canonical NF- $\kappa$ B pathway is activated by diverse stimuli, including the pro-inflammatory cytokines tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-1, antigen receptors or exposure to pathogens like viral and bacterial products. The induction by various stimuli leads to phosphorylation of the I $\kappa$ Bs by the IKK complex, which is composed of the catalytic subunits IKK $\alpha$  and IKK $\beta$  and a non-catalytic scaffolding subunit IKK $\gamma$ /NEMO, leading to ubiquitination and degradation of I $\kappa$ Bs, and the nuclear translocation of the Rel-heterodimers (Silverman and Maniatis, 2001). The noncanonical NF $\kappa$ B pathway is activated in response to TNF superfamily ligands leading to activation of NF $\kappa$ B inducing kinase (NIK) which phosphorylates IKK $\alpha$ , which in turn phosphorylates the precursor p100 and its cleavage by the proteasome to p52 (Xiao G et al, 2001). NIK stability, and hence p100 processing, is regulated by an E3 ubiquitin ligase complex consisting of TRAF2, TRAF3, cIAP1 and cIAP2 (Zarnegar BJ et al, 2008).

The pro-inflammatory canonical pathway is NEMO or IKK $\gamma$  dependent, while the non-canonical pathway depends on NF $\kappa$ B inducing kinase (NIK) and functions independent of IKK $\gamma$ .

The non-canonical pathway is activated in response to tumor necrosis factor (TNF) receptors during lymphoid development (Sun SC, 2011). The crucial role of NFκB proteins in regulation of these pathways is the reason that cancers and viral pathogens exploit NFκB to enhance proliferation, cell survival, and evasion of immune responses.

#### *HTLV-1 Tax deregulation of NFκB and leukemogenesis*

A characteristic feature of ATL is the increased expression of lymphokines and lymphokine receptors (products of genes controlled by NFκB) (Arima et al, 1996; Maruyama et al, 1987; Siekevitz et al, 1987). Tax deregulates the expression of NFκB target genes that play a key role in T-cell growth and survival shortly after establishment of HTLV-1 infection and proviral integration. These genes include growth cytokines like IL-2, IL-15 and their receptors as well as anti-apoptotic genes like BclXL, survivin etc (Sun SC and Ballard DW, 1999). Tax also stimulates NFκB to induce cell-cycle regulators such as cyclin D2 and Cdk6 causing irregular cell-cycle progression. Taken together when these factors are induced continuously in the infected cells, it results in an unstable genomic environment that predisposes them to leukemic transformation.

Tax plays a very important role in HTLV-1 associated leukemogenesis by driving cellular proliferation, cell cycle dysregulation, enhancing cell survival and promoting genetic instability. Mouse models with tax expression in their lymphocyte compartment have constitutively active NFκB and reproduce many aspects of HTLV-1 pathogenesis (Nerenberg MI, 1990). It has been seen that tax expression alone without any other viral factors is sufficient to activate the NFκB pathway and cause leukemia, lymphoma, solid tumors, splenomegaly, and osteolytic disease. In 1990, Nerenberg identified that NFκB is involved in tumor growth in a transgenic mouse model and Tax under the regulation of the HTLV-1 LTR, leads to the development of neurofibromas

(Nerenberg MI, 1990). Another manifestation of LTR driven Tax expression and NFκB pathway activation in mouse models was chronic inflammation, hence in order to better understand ATL-like malignancies, other promoters were used in transgenic constructs to restrict tax expression in the lymphoid compartments. Transgenic mice with Granzyme B promoter had tax expression selectively in activated T-cells and NK cells and developed leukemia/lymphoma and tumors associated with high levels of NFκB and NFκB-regulated genes. This animal model demonstrates that expression of Tax in the lymphocyte compartment is sufficient for the development of leukemia (Grossman WJ et al, 1995). Furthermore, it was shown in two independent studies that Tax expression using an Lck-promoter leads to constitutively elevated NFκB and an ATLL-like malignancy in transgenic mice (Hasegawa H et al, 2006). The persistent expression of NFκB responsive genes in the presence of Tax has been considered to lead to initiation and/or maintenance of the malignant phenotype in HTLV-1 infected cells. It was seen that the inhibition of NFκB activity by using antisense arrest curbs the growth of Tax transformed cells in vitro and in vivo (Kitajima et al, 1992). Most of the studies using Tax mutants defective for either NFκB or CREB/ATF activation were able to prove the association between Tax activation of NFκB and T-cell transformation. And finally, HTLV-1 molecular clones with a single point mutation in Tax oncogene that render it unable to activate NFκB pathway were used to demonstrate that such mutant clones disrupt the ability of the virus to transform primary cells (Robek M and Ratner L, 1999). These data confirmed the role of NFκB in Tax-mediated transformation and also establish that constitutive activation of the NFκB pathway is essential in the process of Tax-mediated oncogenesis.

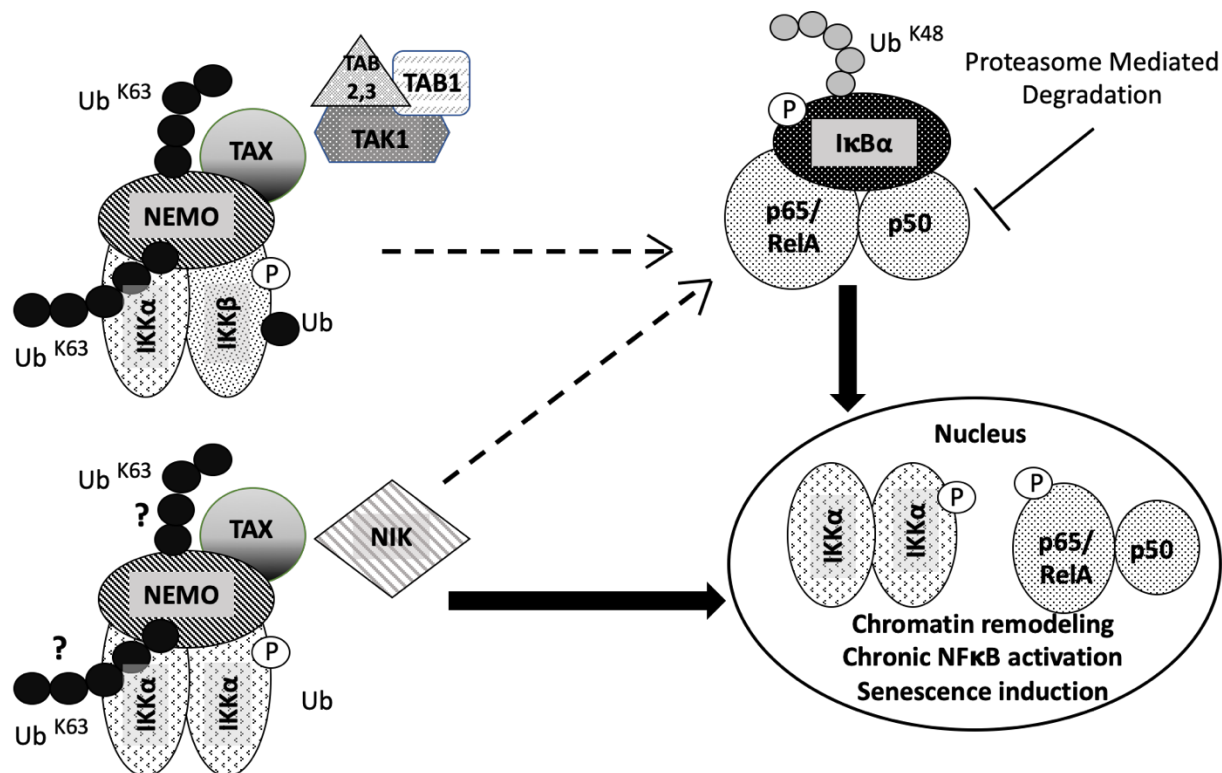
### *Mechanism of Tax activation of NFκB*

Tax acts at multiple levels to induce NFκB activation. Tax is known to stimulate persistent nuclear expression of Rel/NFκB members including p50, p52, Rel-A and c-Rel (Arima et al, 1991). Initially, immunoprecipitation experiments were conducted to show that Tax interacts physically with members of Rel/NFκB and IκB families (Suzuki et al, 1994; 1995). These experiments led to the idea that Tax either dissociates or disrupts the assembly of the p50/RelA/IκB complex. In line with the the dissociation model, Watanabe et al showed that p105 acts as an IκB inhibitor and sequesters p50 and p65 in the cytoplasm and that Tax inhibits the IκB activity of p105, leading to increased nuclear localization of p50 and p65 (Watanabe et al, 1993). Tax also potentially binds to IκBα and dissociates IκBα/NFκB complex and after site-specific phosphorylation and ubiquitination of IκBα targets it to 26S proteasome for degradation thus preventing its inhibitory action on RelA and c-Rel (Suzuki et al, 1994; 1995; Sun SC and Ballard DW, 1999). Moreover, the mutants of IκBα lacking Ser-32 and Ser-36 are completely resistant to Tax-induced degradation and are able to repress Tax. The two serines Ser-32 and Ser-36 are phosphorylated for targeting IκBα to the proteasome in cells activated with the pro-inflammatory cytokines TNFα or IL-1. Thus it can be elucidated that by a phosphorylation-dependent mechanism Tax converts both IκBα and IκBβ into degradable proteasome substrates and lead to persistent NFκB activation. (Sun SC and Ballard DW, 1999).

Three observations led to deduction that cytokine-responsive IKKs are the primary cellular targets of Tax in the NFκB pathway. First, Tax associates with constitutively active IKKs to form dissociation resistant higher order complexes in vitro. Second, it was seen that the kinase-inactive



forms of IKK $\alpha$  and IKK $\beta$  interfere with Tax-mediated NF $\kappa$ B activation. Finally, the mutants of Tax that are unable to interact with IKKs do not activate NF $\kappa$ B (Hiscot J et al, 2001).



**Figure 4. Schematic representation of the possible mechanisms by which Tax activates NF $\kappa$ B pathway.** Tax interacts with and recruits NEMO (IKK $\gamma$ ) and then recruits TAK1 and NIK to the IKK complex to initiate the canonical pathway and to some extent also the non-canonical pathway. Tax recruits TAK1 to IKK possibly by K63-linked poly ubiquitination, however if NIK is recruited by K63 poly ubiquitination is not clear. Nuclear localization of p65/RelA and IKK induces chromatin remodeling and target gene activation along with NF $\kappa$ B activation, possibly leading to senescence. (adapted from Yik-Khuan Ho et al, 2012)

In the cytoplasm, the Tax dimers activate IKK by interacting with the non-catalytic subunit IKK $\gamma$  (NEMO) which allows the recruitment of Tax to the catalytic subunits IKK $\alpha$  and IKK $\beta$ . This interaction and activation of IKK by Tax depends on its conjugation to Ub-K63 chains formed by Ubc13 and removed by USP20. The first evidence linking IKK $\gamma$  and Tax emerged from genetic

complementation experiments where an IKK $\gamma$ -deficient rat fibroblast line fails to express constitutively active forms of NF $\kappa$ B in the presence of Tax. This loss of NF $\kappa$ B signaling correlated with a reduced molecular size of IKK complexes and overexpression of IKK $\gamma$  was able to rescue the phenotype, suggesting that IKK $\gamma$  is required for the proper assembly of the Tax-responsive IKK complex (Yamaoka et al, 1998). Moreover, inhibition of IKK $\gamma$  by antisense approach in human T-cells inhibited Tax-mediated activation of NF- $\kappa$ B but did not affect CREB/ATF (Hiscot J et al, 2001). Mutant T-cell lines deficient in IKK $\gamma$  expression had no NF $\kappa$ B activation when stimulated by both T-cell costimulatory signals and HTLV-1 Tax but the NF $\kappa$ B signaling was restored with exogenous IKK $\gamma$  (Harhaj EW et al, 2000). Overexpression of Tax, IKK $\gamma$  and either IKK $\alpha$  or IKK $\beta$  in vivo leads to a measurable I $\kappa$ B kinase activity but in the absence of IKK $\gamma$  there is no induction of its kinase activity showing the indispensable role of IKK $\gamma$  as a molecular adaptor that provides a site for Tax binding and the assembly of Tax/IKK complexes (Hiscot J et al, 2001).

Tax can also interact with upstream kinases such as MEKK1, NIK, optineurin, TAK1, and PIN1 and activate IKK by phosphorylation, ubiquitination and proteasome mediated degradation of I $\kappa$ Bs (Kafoury Y et al, 2010). This I $\kappa$ B-degradation exposes the NLS of NF $\kappa$ B dimers, allowing their rapid translocation to the nucleus and NF $\kappa$ B activation. Tax can also disrupt the Ubc13/A20 complex through the interaction with Tax1BP1 making Ubc13 available for its ubiquitination (Shembade et al, 2008; 2010). The phosphorylation, ubiquitination and proteasomal degradation of I $\kappa$ B $\alpha$  play a key role in activation of NF $\kappa$ B pathway by Tax. Tax has no kinase activity of its own and it binds to the IKK complex (Sun SC and Ballard DW, 1999), causing a direct interaction with the IKK $\gamma$  - the IKK regulatory subunit NEMO (Kafoury Y et al, 2010). This direct interaction allows the recruitment of catalytic IKK $\alpha$  and IKK $\beta$  subunits. In order to activate the non-canonical NF $\kappa$ B pathway, Tax requires NEMO and IKK $\alpha$  and forms a complex with p100 causing

phosphorylation dependent ubiquitination and processing of p100 to p52 (Xiao et al, 2001). Thus, by targeting different IKK complexes, Tax activates both canonical and non-canonical NFκB pathways.

In ATL cells, just like in normal T-cells, NFκB activation has been shown to prevents apoptosis. Thus, constitutive activation of NFκB pathway by HTLV-1 Tax leads to aberrant growth and cytokine gene expression. A large body of data indicates that this constitutive NFκB activation by the virus is required to maintain and/or induce the transformed phenotype of HTLV-1 infected cells.

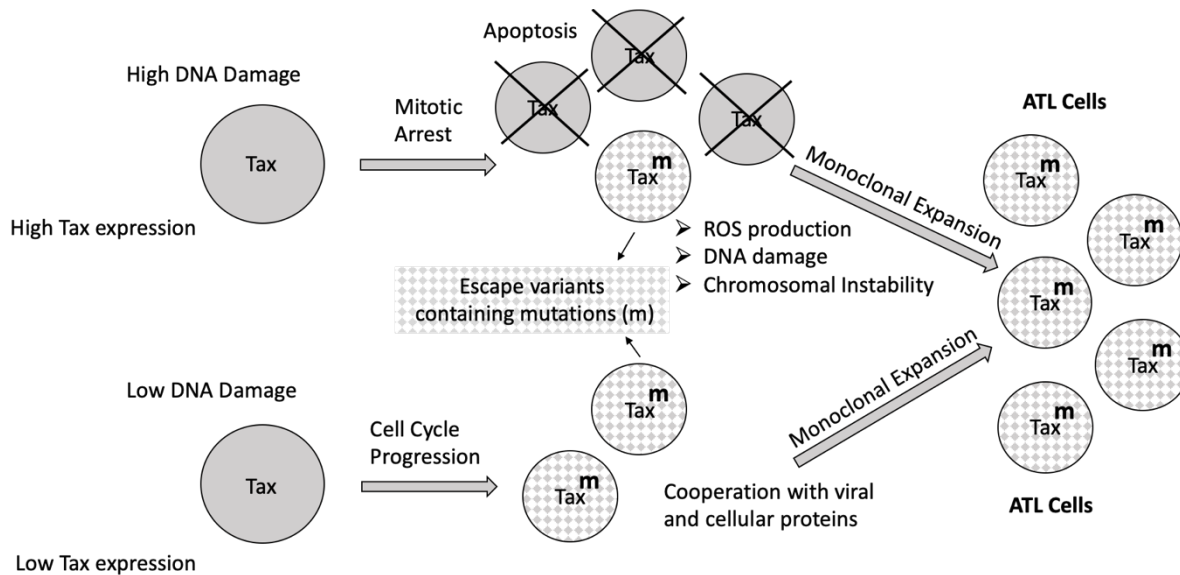
#### **1.4 Genomic instability and DNA damage repair pathways in HTLV-1**

Cancer is a genetic disease. DNA damage leads to errors during DNA synthesis causing mutations that may lead to cancer. In eukaryotic organisms a DNA damage response pathway has been developed to protect against such insults to the genome. The DNA damage response causes cell cycle arrest and allows for DNA repair functions in cells with minor damages which ultimately survive. However, the cells with severe DNA damage are directed to undergo apoptosis. Tax is expressed at low levels in ATL cells and has a weak oncogenic activity, but the genomic and genetic instability caused by Tax plays a key role in the development of ATL (Baydoun H et al, 2012). In HTLV-1 transformed cells and Tax expressing cells clastogenic DNA damage including point mutations, deletions, substitutions, translocations and rearrangements, are frequently seen (Marriott S et al, 2002). Tax has the ability to not only suppress DNA repair, but in addition it can stimulate cell cycle progression and block apoptosis resulting in the introduction of mutations into the genome. Owing to these combined biological effects of Tax, a 2.8-fold increase in genomic mutation frequency had been demonstrated (Miyake H et al, 1999).

HTLV-1 Tax can inhibit the cells ability to repair DNA damage resulting in accumulation of mutations in infected cells (Jeang KT et al, 1990; Marriott S et al, 2002; Kao S-Y et al, 1999). It is common for most cells to acquire DNA damage as they transit the cell cycle. However, in most normal cells, the DNA damage that exists in the first growth phase, G1, of the cell cycle can be repaired by DNA repair mechanisms such as base excision repair (BER), nucleotide excision repair (NER), recombination and direct repair of nicks by DNA ligation. In 1990, Jeang et al, for the first time alluded that HTLV-1 suppresses DNA repair through Tax which decreases BER by repressing the expression of DNA-polymerase  $\beta$  (Jeang KT et al, 1990). HTLV-1 Tax was shown to suppress NER in rat fibroblasts exposed to UV irradiation (Kao S-Y et al, 1999). UV irradiation of cells usually leads to NER which uses DNA polymerases  $\delta$  and  $\epsilon$  and a cofactor PCNA (proliferating cell nuclear antigen) for its repair activity. Tax can cause excess PCNA synthesis by inducing its up-regulation by transactivating the PCNA promoter (Lemoine FJ et al, 2000). In presence of excess PCNA, DNA pol  $\delta$  inappropriately synthesizes excess new DNA going past the template lesion resulting nucleotide mis incorporation (Lemoine FJ et al, 2000; Marriott S et al, 2002).

An early DNA double-strand breaks sensing mechanism is the activation of ataxia telangiectasia mutated (ATM) kinase. ATM kinase sends the DNA damage signal to cell cycle checkpoint control which facilitates DNA damage repair by activating repair proteins. In the presence of Tax expression, ATM kinase activity is prematurely attenuated and doesn't allow recruitment of MDC1 to the DNA repair site rendering ATM inactive. Thus, in Tax expressing cells, ATM is not able to facilitate DNA damage repair, resulting in premature DNA replication along with the present genomic lesion leading to accumulation of mutations (Chandhasin C et al, 2008).

Preneoplastic cells proliferate at a high rate in order to accumulate genetic mutations and acquire a transformed phenotype. Each cell division involves progressive shortening of telomeres which can initiate senescence and irreversible cell cycle arrest or cell death after a finite number of cellular divisions. This mechanism can suppress tumor development. In about 90% of cancers, tumor cells overcome this barrier by reactivating telomerase enzyme (Chlichlia K and Khazaie K, 2010). Telomerase activity is increased at the post-transcriptional level by AKT, the p65 subunit of NF $\kappa$ B, SHP2, 14-3-3 protein, and PKC-mediated phosphorylation. Since there are high levels of AKT and NF $\kappa$ B in HTLV-I cells, it is possible that these pathways could be very important in maintaining the high telomerase activity seen in these cells (Bellon M and Nicot C, 2015). Normally, telomeric repeats at the chromosomal ends prevent aberrant end-to-end chromosomal fusions, stabilize de novo double strand chromosomal breaks, and also protect them from degradation by endonucleases. Recent evidence also suggests that Tax represses the transcription and expression of human telomerase (hTERT) resulting in telomerase inactivation in Tax expressing cells. Tax prevents the addition of telomeric repeats to new double-stranded breaks and potentially interferes with a protective mechanism used to prevent inappropriate chromosomal breakages-fusions (Jeang KT et al, 2004). On the contrary, it was also shown that in the absence of exogenous mitogenic stimulation, Tax is able to stimulate the hTERT promoter through the NF $\kappa$ B pathway which may result in transient genetic instability during mitosis (Gabet A-S et al, 2003). Even when the mitogenic effect has vanished, this tax mediated activation of hTERT gives long term proliferative advantage to these cells that have acquired chromosomal abnormalities. The sum of effects of Tax on BER, NER, DNA end stability, telomerase, and cell cycle progression lead to a compromised state of DNA damage repair in the cells termed as the mutator phenotype and is a critical component of cellular transformation.



**Figure 5. Schematic representation of Tax expression** in cells leading to either apoptosis or cell cycle progression and contributing to transformation (adapted from Chlichlia K and Khazaie K, 2010)

In order to maintain the diploid state in human cells, it is required that a precise and reproducible equal chromosomal division occurs from mother to two daughter cells. Majority of cancers and transformed cells typically present numerical chromosomal changes including loss or gain of entire chromosomes (aneuploidy) owing to unequal chromosomal division, in addition to other structural chromosomal alterations. ATL cells are no different in this respect and have highly lobulated “flower” shaped nuclei. Mitotic spindle assembly checkpoint (MSC) is the cellular mechanism that guards against chromosome missegregation and maintains the state of euploidy (Marriott S et al, 2002). Several ATL cell lines were found to be deficient in MSC function (Kasai T et al, 2002). At least seven genes have been identified for MSC function in yeast and mammals including Mps1 (monopolar spindle), BUB 1, 2 and 3 (budding uninhibited by benomyl) and MAD 1, 2 and 3 (mitotic arrest deficient) (Marriott S et al, 2002). In events of spindle damage or

segregation anomalies the MSC is activated to arrest metaphase to anaphase transition and prevents cell division and initiates apoptosis curbing the propagation of chromosomal abnormality. MAD2 is the distal effector protein of metaphase-anaphase checkpoint. MAD2 binds to CDC20 and inhibits the function of APC (anaphase promoting complex). Tax binds human MAD1 (hsMAD1) which is an integral constituent of MSC. hsMAD1 is required to direct MAD2 to kinetochores in the nucleus where it is critical to carry normal MSC function. This association between Tax and hsMAD1 causes MAD1 and MAD2 to translocate to the cytoplasm from the nucleus leading to loss of MSC function and M phase to proceed uninterrupted ultimately resulting in aneuploidy. (Kasai T et al, 2002). Tax also interacts with TAX1BP2, a centrosomal protein which normally blocks centriole replication, and causes multipolar mitosis which is another common cause of aneuploidy (Ching YP et al, 2006). Furthermore, Tax interacts with RANBP1 during mitosis and causes fragmentation of spindle poles, thereby promoting multipolar mitosis and asymmetrical chromosome segregation (Peloponese JM et al, 2005). These mechanisms involving Tax interactions with TAX1BP1 and RANBP1 explain the frequent occurrence of aneuploidy and multipolar spindles in ATL cells.

Baydoun et al showed that Tax-mediated NF $\kappa$ B activation is important for inhibition of homologous recombination (HR) DNA repair. They used the ACH clones (ACH.wt and ACH.p30<sup>II</sup>) and also Tax mutants M47 and M22 and saw that M47 which is defective for NF $\kappa$ B pathway activation significantly inhibited HR but not the M22 mutant which can activate NF $\kappa$ B robustly (Baydoun HH et al, 2012). This study concluded the role of Tax in inhibiting the DDSB HR repair through activating the NF $\kappa$ B pathway.

It is possible that NF $\kappa$ B may also contribute to oncogenesis through DNA damage and induction of mutations indirectly through inflammation-mediated production of reactive oxygen

and nitrogen species (ROS and RNS) (Xiao G and Fu J, 2011). More recently, it was shown that HTLV-1 infected T-cells and cells expressing Tax exhibit high levels of intracellular nitric oxide (NO). NO and its product peroxynitrite are highly reactive molecules that cause DNA damage. iNOS is the enzyme that catalyzes the production of NO in T-cells, macrophages and neutrophils. Tax activates iNOS and induces DSBs through the induction of NO by the classical NF $\kappa$ B and JAK/STAT pathways, which are persistently activated in the proliferation of HTLV-1 infected T-lymphocytes (Baydoun HH et al, 2015). Chaib-Mezrag et al showed that Tax induces DDSBs through activation of NF $\kappa$ B pathway and by stimulating intracellular NOS. They also showed that in the presence of Tax, the replication forks are slower leading to genomic defects and increased DDSBs (Chaib-Mezrag H et al, 2014). Taken together, Tax targets proteins involved in DNA replication, DNA damage and repair, centrosome duplication, and cell cycle checkpoints leading to genomic instability and acquisition of mutations, paving the way for leukemogenesis.

### **1.5 Stathmin – Oncoprotein 18 (OP18)**

Stathmin also known as oncoprotein 18 (OP18), is a 149 aa long cytosolic phosphoprotein integrating several intracellular pathways involved in cell division, cell motility and intracellular transport (Curmi AP et al, 1999). Stathmin expression is very high in early embryonic stages in the undifferentiated cells but it strongly decreases in the adult tissue except in nervous system and in testis (Curmi AP et al, 1999). However, stathmin overexpression has been reported in several types of cancers including acute leukemia, lymphomas, cervical cancer, breast cancer, prostate cancer, lung cancer, osteosarcoma, and gastrointestinal tumors including pancreatic cancer (Lu Y et al, 2014). Microtubules exist as long polymers and form the cytoskeleton of eukaryotic cells. Stathmin has been found to interact with tubulin and control microtubule dynamics. It can destabilize microtubules by sequestering tubulin heterodimers and directly promoting catastrophes



leading to a decrease in the amount of free tubulin for microtubule assembly (Charbaut E et al, 2001). Microtubule disruption may lead to formation of abnormal mitotic spindle, inaccurate chromosomal segregation, attenuated cell motility and possibly facilitate cancer development. Some previous studies have confirmed the oncogenic role of stathmin in promoting cancer cell proliferation, differentiation, migration and invasion (Lu Y et al, 2014). Any mutation or aberrantly expressed protein that compromises the fidelity of spindle assembly increases chromosomal instability and is considered to be aneugenic since such defects contribute to aneuploidy. A hyperactive Q18E Stathmin mutant, or overexpression of wildtype Stathmin, has been reported to cause aneugenic effects and chromosomal instability linked to ploidy changes in leukemic cells (Holmfeldt et al, 2006). Overexpression of wild-type Op18 results in aneugenic activities, leading to aberrant mitosis, polyploidy, and chromosome loss. It was seen that the aneugenic activity of Op18 was dramatically increased by the somatic Q18→E missense mutation (Holmfeldt et al, 2006). Tubulin assembly studies in vitro have determined that the N-terminal nonhelical region of Op18 is essential for catastrophe promotion, whereas the tandem helical repeats are required for tubulin-sequestering activity (Howell et al, 1999). Human Op18 can be inactivated to various degrees by differential phosphorylation at four Serine residues (Ser-16, -25, -38, and -63) by cell cycle-regulating and signal-transducing kinase systems. Although Op18 is phosphorylated at multiple sites during mitosis, it exists predominantly in its unphosphorylated active form during interphase (Brattsand et al, 1994; Larsson et al, 1997).

Stathmin is transcriptionally repressed by wild-type p53. p53 forms a transcriptional repression complex with HDAC and mSin3a where mSin3a binds to the promoter of stathmin and reduces its association with acetylated histone H3 resulting in repression of transcription in the presence of wild-type p53 (Murphy M et al, 1999). In normal cells, p53 tumor suppressor protein is critical in

cellular response to stress and leads to increased p53 protein levels which translocates to the nucleus to act as a transcription factor activating genes associated with either growth arrest or apoptosis (Levine et al, 1997). The mutation or loss of p53 activity is one of the most frequent events in human cancer (Hollstein et al, 1994). Unlike most cancers however HTLV-1 positive ATLL clinical isolates mostly have a high level of wildtype p53 (Zane L et al, 2012). HTLV-1 latency maintenance factor p30<sup>II</sup> has been shown to interact with MYST-family acetyltransferase TIP60 resulting in inhibition of K120-acetylation of the p53 protein (Romeo MM et al, 2018) leading to regulation and expression of p53 regulated pro-apoptotic genes (Sykes SM et al, 2006). Murphy et al proposed a possibility that in addition to contributing to trans-repression of genes like stathmin and Map4, the HDAC–mSin3a–p53 association may also result in the deacetylation of p53 and have a role in the regulation of this protein (Murphy M et al, 1999). This alludes to the possibility that in ATL, with majority of wildtype p53, the p53-target genes are subverted selectively to promote oncogenesis (Romeo MM et al, 2018).

Successful chemotherapy treatments such as using paclitaxel and vinblastine are believed to act by targeting microtubules. They disrupt the spindle function, activate spindle assembly checkpoint and induce cell death. More recently, novel chemotherapy targets such as microtubule regulatory proteins including stathmin/OP18 have received attention (Rana S et al, 2008). Moreover, high stathmin or phosphorylated stathmin level in tumor tissues predicts a poor overall survival and was described as a potential biomarker for metastasis or local recurrence in some human cancers (Lu et al, 2014). p27kip1 is a cell cycle inhibitor and also a regulator of cell motility (Belletti B et al, 2010). p27 gets phosphorylated on threonine residue T198 which allows it to bind stathmin in the cytoplasm and inhibits its microtubule destabilizing activity (Schiappacassi M et al, 2011). In the absence of p27, there is a decrease in microtubule stability which promotes

increased motility and invasive potential of cells. Interestingly, HTLV-1-transformed cells have very low levels of p27 (Kuo YL and Giam CZ, 2006) which may allow abundant stathmin binding to and destabilizing microtubules and aberrant mitosis leading to genomic instability in ATL cells.

Lu et al found a correlation between stathmin and NF $\kappa$ B pathway in pancreatic cancer. They established that stathmin expression correlated with poor prognosis of pancreatic cancer and stathmin promotes pancreatic cancer cell proliferation through its interaction with p65<sup>RelA</sup>. In this study they confirmed that NF $\kappa$ B/p65<sup>RelA</sup> was downregulated when stathmin was knocked-down and the CoIPs showed an interaction between Stathmin and p65<sup>RelA</sup> suggesting a novel oncogenic role of stathmin in aggressive pancreatic cancer (Lu et al, 2014).

### **1.6 Lipid Rafts – Stathmin – TIGAR**

Lipid rafts are cholesterol- and sphingolipid-rich, detergent-resistant plasma membrane microdomains. Lipid rafts are synthesized from the Golgi and are recycled between the Golgi and the plasma membrane (Rocks O et al, 2005). These lipid raft microdomains are crucial membrane structures and often serve as signal transduction platforms for mediating kinase activation in TCR signaling (Cheng H et al, 2010). Some researchers have proposed lipid rafts to be associated with cytoskeleton from experiments conducted in rat forebrain extracts and in human smooth muscle cells (Dremina ES et al, 2005, Kawabe J et al, 2006). In fact, some reports indicated that when microtubule disrupting agents such as colchicine were used on glias or cardiomyocytes, it resulted in the loss of signaling molecules from lipid rafts (Jin S et al, 2011).

The persistent activation of NF $\kappa$ B by HTLV-1 Tax is a characteristic feature of ATL and plays a crucial role in T-cell transformation and ATL development. Tax has been shown to induce NF $\kappa$ B activation through several different mechanisms. One of those is through the recruitment of IKK

signalosome to the lipid raft microdomains. Tax is considered to be a lipid raft modulator that hijacks IKKs to the lipid rafts for the activation of NF $\kappa$ B through physical interaction with IKK $\gamma$  and promotes T-cell proliferation (Huang J et al, 2009). Ren T et al showed that Tax can also utilize this I $\kappa$ B kinase dependent NF $\kappa$ B activation in lipid rafts to promote autophagy by recruiting the autophagic complex to the lipid raft microdomains. Their research further suggests the possible role of Tax in utilizing lipid rafts as signaling platform to recruit I $\kappa$ B kinases and autophagy molecules into these lipid raft microdomains and activating NF $\kappa$ B and autophagy pathways (Ren T et al, 2015). It is however known that Tax does not contain a consensus lipid raft signal peptide and it is plausible that Tax may interact with a cellular lipid raft-associated protein for targeting to the lipid raft domains.

Microtubules provide a polarized network in the cells that allows organelle and protein movements. Microtubules have been shown to regulate membrane trafficking and lipid raft localization of certain signaling components (Head BP et al, 2006). Lipid raft components have been shown to move throughout the cell cytoplasm via cytoskeletal structural scaffold and can contribute to dynamic organelle structural remodeling (Sorice M et al, 2010). Moreover, it was observed that disruption of microtubules leads to loss of lipid raft caveolae and redistribution of lipid raft resident proteins and may ultimately lead to disruption of certain signaling pathways associated with lipid rafts (Head BP et al, 2006). Interestingly, stathmin is a microtubule associated protein which, if uninhibited, is involved in disrupting tubulin polymers and can cause microtubule catastrophe (Curmi PA et al, 1999). Belletti et al also showed that p27<sup>kip1</sup> and stathmin interaction regulates lipid raft trafficking and control cell migration, invasion and division (Belletti B et al, 2010). Stathmin activity has also been linked to vesicular trafficking regulation in T-helper cells where it plays a role in controlling interleukin-4 receptor recycling (Tanaka et al, 2007). This

alludes to a possible role of stathmin in disrupting lipid raft associated signaling by microtubule regulation and leading to dampened NF $\kappa$ B activation in the lipid rafts microdomains of HTLV-1 infected T-cells.

TIGAR (TP53-induced glycolysis and apoptosis regulator), is a p53-target gene that affects ROS by modulating the glycolytic pathway and protects the cell against oxidative stress (Bensaad et al, 2006). TIGAR protein has similarity to the bisphosphatase domain of phosphofructo-2-kinase/ fructose-2,6-bisphosphatase (PFK-2/FBPase-2). PFK-2 plays an essential role in glycolysis regulation. Li and Jøgl showed that TIGAR can hydrolyze fructose 2,6-bisphosphate and fructose 1,6-bisphosphate resulting in their decrease and ultimately reducing glycolysis in cell (Li and Jøgl, 2009). In addition to the reduction in glycolysis, TIGAR expression also redirects the glycolytic metabolic intermediates to the oxidative branch of pentose phosphate pathway resulting in an increased production of NADPH. Increased NADPH can scavenge ROS by reduced glutathione leading to decreased intracellular ROS and lower apoptosis resulting due to oxidative stress (Bansaad et al, 2006). TIGAR overexpression has been linked to decreased ROS levels and inhibits autophagy (Bensaad K et al, 2009).

Autophagy is a catabolic process and occurs in response to metabolic stress by sequestering and degrading cellular proteins and organelles in autolysosomes (Baudhuin P, 1966). Autophagy is considered mainly pro-survival and thus autophagy is also known to contribute to chemotherapy resistance (Ren T et al, 2015). Furthermore, in certain tumorigenic viruses autophagy is necessary for their replication and induction of oncogenesis (Ren T et al, 2015). In Hepatitis C virus (HCV) infection autophagy promotes initiation of viral infection, and inhibition of autophagy represses viral replication (Dreux M and Chisari FV, 2009). Latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) induces early or late stage autophagy depending on its expression levels (Lee

DY and Sugden B, 2008). Inhibition of autophagy in EBV-infected cells suppresses its transforming potential. Also, Hepatitis B Virus (HBV) sensitizes cells to starvation-induced autophagy by increasing Beclin1 (BECN1) expression (Tang H et al, 2009). In Hepatitis C virus (HCV) infected cells, an association between lipid rafts and autophagosomes has been confirmed, which the virus uses for its RNA replication (Kim JY et al, 2017). Lipid rafts are associated with autophagy associated molecules and contribute to autophagosome morphogenesis. A recent study by Matarrese et al found that GD3 ganglioside, a key lipid raft constituent, actively contributed to the biogenesis and maturation of autophagic vacuoles in human and murine primary fibroblasts (Matarrese P et al, 2014). Furthermore, the viral LMP1 protein from EBV associates with the lipid raft microdomains where it activates NF $\kappa$ B and also induces autophagy (Lee DY and Sugden B, 2008). Ren T et al found that Tax-deregulated autophagy is associated with the lipid raft recruitment of the autophagic molecular complex containing Beclin1 and Bif-1 which increases LC3<sup>+</sup> autophagosomes and promotes autophagy. They also confirmed that Bif-1 and BECN1, together with IKK and Tax, were constitutively present in the lipid raft fraction in Tax-expressing T cell lines (Ren T et al, 2015). It was also recently demonstrated that p30<sup>II</sup> activates p53 and induces the expression of p53-regulated pro-survival signals, including TIGAR, increasing the oncogenicity of the HTLV-1 (Romeo M et al, 2018; Hutchison T et al, 2018).

Although the exact role of lipid rafts in autophagy induction is presently not clear but there seems to exist a potential association between the two. As mentioned earlier, TIGAR overexpression has been seen to inhibit autophagy (Bensaad K et al, 2009). The possibility that p30<sup>II</sup> induces p53 which in turn upregulates TIGAR in HTLV-1 infected cells leading to suppression of autophagy by TIGAR further leads to inhibition of IKK signaling in lipid rafts and hence dampens Tax induced NF $\kappa$ B activation needs to be addressed.

## CHAPTER II

### EXPERIMENTAL RESULTS AND DISCUSSION

#### **2.1 HTLV-1 latency maintenance protein p30<sup>II</sup> inhibits TIP60 dependent K120-acetylation of p53 and represses Stathmin/OP18 expression**

Rationale: The mutation or loss of p53 activity is one of the most frequent events in human cancer (Hollstein et al, 1994). However, unlike most cancers, HTLV-1 positive ATLL clinical isolates mostly have a high level of wildtype p53 (Zane L et al, 2012). The p53 transcription factor gets activated in response to stimuli such as DNA damage, hypoxia or cellular and viral oncogenes. In response to these stimuli, p53 either induces or represses a set of p53-response genes and regulates the expression of various pro-survival or pro-apoptotic pathway genes (Murphy M et al, 1999). p53 post-translational modifications can modulate its functions and can dictate its response to various cellular signals. Acetylation of p53 protein increases its stability, activation, binding, antiviral activities and also plays a role in DNA damage checkpoint response and oncogene activation response (Murphy M et al, 1999). In general, p53 acetylation is mediated by two different acetyltransferases, p300/CBP/PCAF or Tip60/MOF/MOZ on one or more seven C-terminal lysines, leading to p53-dependent activation of apoptosis and senescence (Brooks C and Gu W, 2011). This indicates that acetylation is a critical regulator of p53 anti-cancer functions. Previous studies from our lab have shown that the HTLV-1 latency maintenance factor p30<sup>II</sup> protein cooperates with cellular oncoproteins and induces activation of p53-dependent pro-survival genes leading to aberrant lymphoproliferation (Romeo M et al, 2014). HTLV-1 p30<sup>II</sup> has been shown to interact with MYST-family acetyltransferase TIP60 resulting in inhibition of K120-

acetylation of the p53 protein (Romeo MM et al, 2018) which causes expression of p53 regulated pro-apoptotic genes. ***To take this further we tested whether p30<sup>II</sup> can cooperate with cMyc to inhibit TIP60 mediated K120 acetylation of p53 and activate pro-survival pathways in HTLV-1 infected cells.***

Stathmin is a microtubule-destabilizing factor and has been shown to play an oncogenic role in a range of invasive cancers (Lu et al, 2014). The stathmin gene is transcriptionally repressed by wild-type p53 through formation of p53/mSin3a/HDAC1 repressor complexes (Murphy M et al, 1999). In aggressive pancreatic tumors Stathmin has been shown to interact with the p65<sup>RelA</sup> subunit of NFκB and it acts as a cofactor for NFκB-signaling by stabilizing the p65<sup>RelA</sup> protein (Lu et al, 2014). Since HTLV-1 infected cells have a high level of wild-type p53 and Stathmin is negatively regulated by p53, there is a possibility that p30<sup>II</sup>, through its induction of p53 (Romeo M et al, 2014) suppresses Stathmin. ***This prompted us to investigate whether HTLV-1 p30<sup>II</sup> inhibits Stathmin/OP18 and p65<sup>RelA</sup>-Stathmin/Op-18 molecular interactions owing to the negative regulation of Stathmin by p53-repressor complexes.***

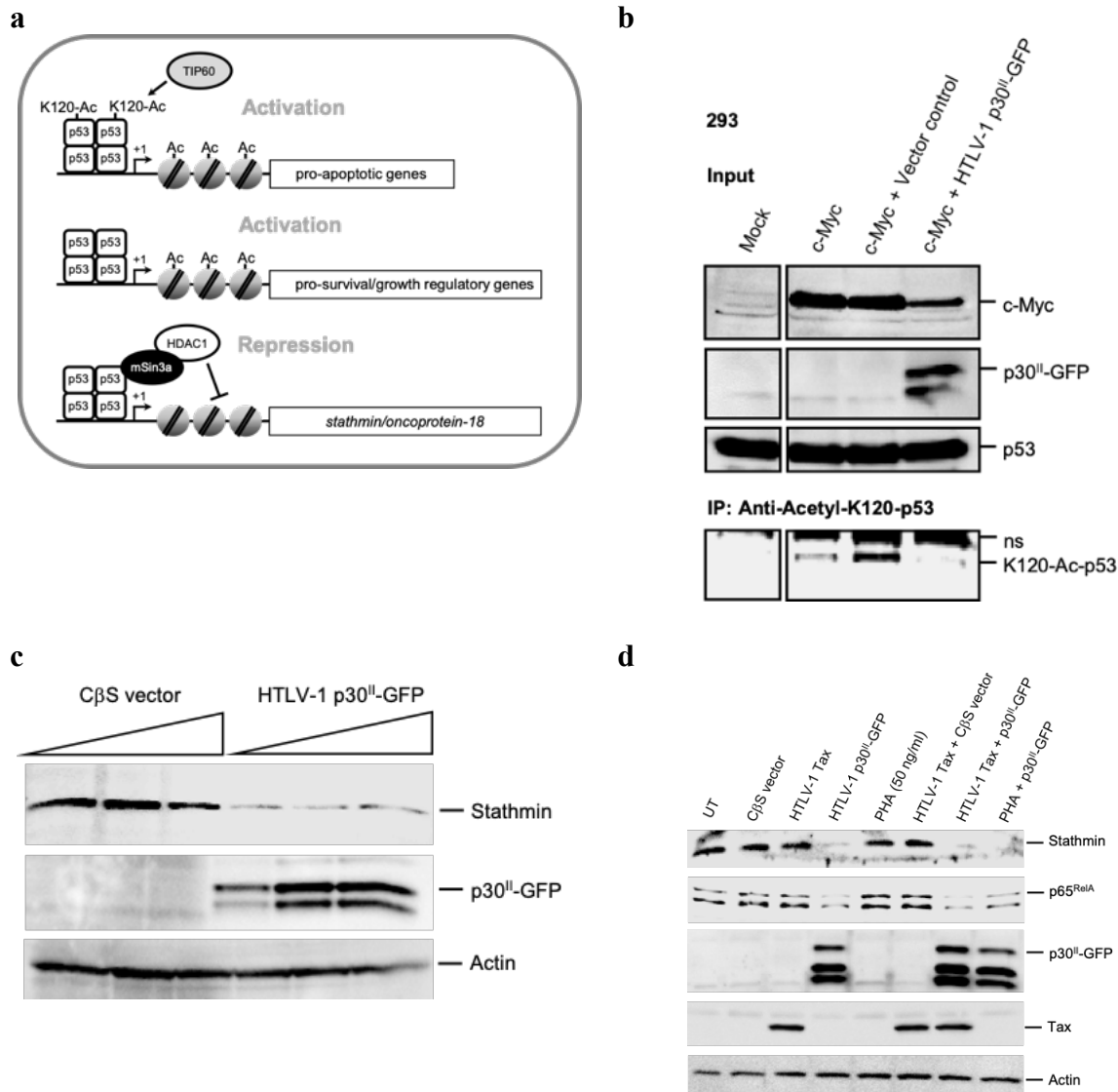
Result and Discussion: HTLV-1 p30<sup>II</sup> can interact with the histone acetyl transferase (HAT) TIP60 (Awasthi S et al, 2005; Romeo M et al, 2015). Awasthi et al (2005) showed that in the presence of HTLV-1 p30<sup>II</sup>, c-Myc is acetylated by the HAT TIP60. Romeo et al (2015) confirmed that p30<sup>II</sup> enhances c-Myc-induced cellular transformation which is dependent on lysine acetylation through the cofactor TIP60. Moreover, Romeo et al (2014) showed that p30<sup>II</sup> activates the expression of p53 in the presence of c-Myc oncoprotein. In order to assess the effect of p30<sup>II</sup> and c-Myc cooperation on p53 K120 acetylation, co-immunoprecipitation studies were performed in 293 HEK cells. 293 HEK cells were transfected with wild-type FLAG-tagged c-Myc and an empty vector control and showed high p53-K120 acetylation through immunoblotting (Fig. 6b).



In the assay, 293 HEK cells were also transfected with wild-type FLAG-tagged c-MYC and CMV-HTLV-1 p30<sup>II</sup>-Green fluorescent protein fusion (p30<sup>II</sup>-GFP) showing no acetylation through the immunoprecipitation with the anti-acetyl-K120-p53 antibody (Fig. 6b). This experiment revealed that HTLV-1 p30<sup>II</sup> protein activates p53 and inhibits TIP60-mediated K120 acetylation of p53 (Fig. 6b). This inhibition of K120 acetylation of p53 can differentially regulate p53 dependent pro-apoptotic gene expression and promote aberrant lymphoproliferation in ATL cells (Sykes SM et al, 2006). Since Stathmin/Op18 is one of the few genes that are negatively regulated by p53 (Murphy M et al, 1999), we wanted to see the effect of p30<sup>II</sup> expression on stathmin. 293 HEK cells were transfected with increasing concentrations of either the empty vector or p30<sup>II</sup>-GFP. Clearly, the p30<sup>II</sup>-GFP protein inhibited Stathmin protein expression as compared to empty C $\beta$ S vector in 293 HEK cells (Fig. 6c). The p30<sup>II</sup>-GFP was detected by immunoblotting and relative Actin protein levels were also detected for comparison (Fig. 6c). Since Stathmin has been shown to interact with the p65<sup>RelA</sup> subunit and acts as a cofactor for NF $\kappa$ B-signaling by stabilizing the p65<sup>RelA</sup> protein (Lu et al, 2014), we tested whether p30<sup>II</sup> might affect p65<sup>RelA</sup> stability by inhibiting the cofactor stathmin. 293 HEK cells were transfected with either empty vector, p30<sup>II</sup>-GFP, HTLV-1 Tax or phytohemagglutinin (PHA)-stimulated. PHA is a known NF $\kappa$ B activator in human cells. 293 HEK cells were also co-transfected with HTLV-1 Tax and C $\beta$ S vector, HTLV-1 Tax and HTLV-1 p30<sup>II</sup>-GFP and HTLV-1 p30<sup>II</sup>-GFP and PHA stimulation. The results in figure 6d indeed showed that repression of Stathmin also destabilized the NF $\kappa$ B subunit p65<sup>RelA</sup> in cells expressing CMV-HTLV-1 Tax or in PHA-stimulated cells (Fig. 6d). These findings are consistent with Lu et al's study (Lu et al, 2014) confirming the role of Stathmin as a cofactor for p65<sup>RelA</sup>.

Overall these results indicate that HTLV-1 p30<sup>II</sup> inhibits TIP60 mediated p53-K120 acetylation leading to subversion of p53 pro-apoptotic pathways. p30<sup>II</sup> also inhibits Stathmin/OP18 owing to

its negative regulation by p53 and this repression of stathmin by p30<sup>II</sup> also destabilizes p65<sup>RelA</sup> subunit of NFκB. This alludes to the possibility that HTLV-1 p30<sup>II</sup> might counter the Tax-induced hyperactivation of NFκB-signaling.



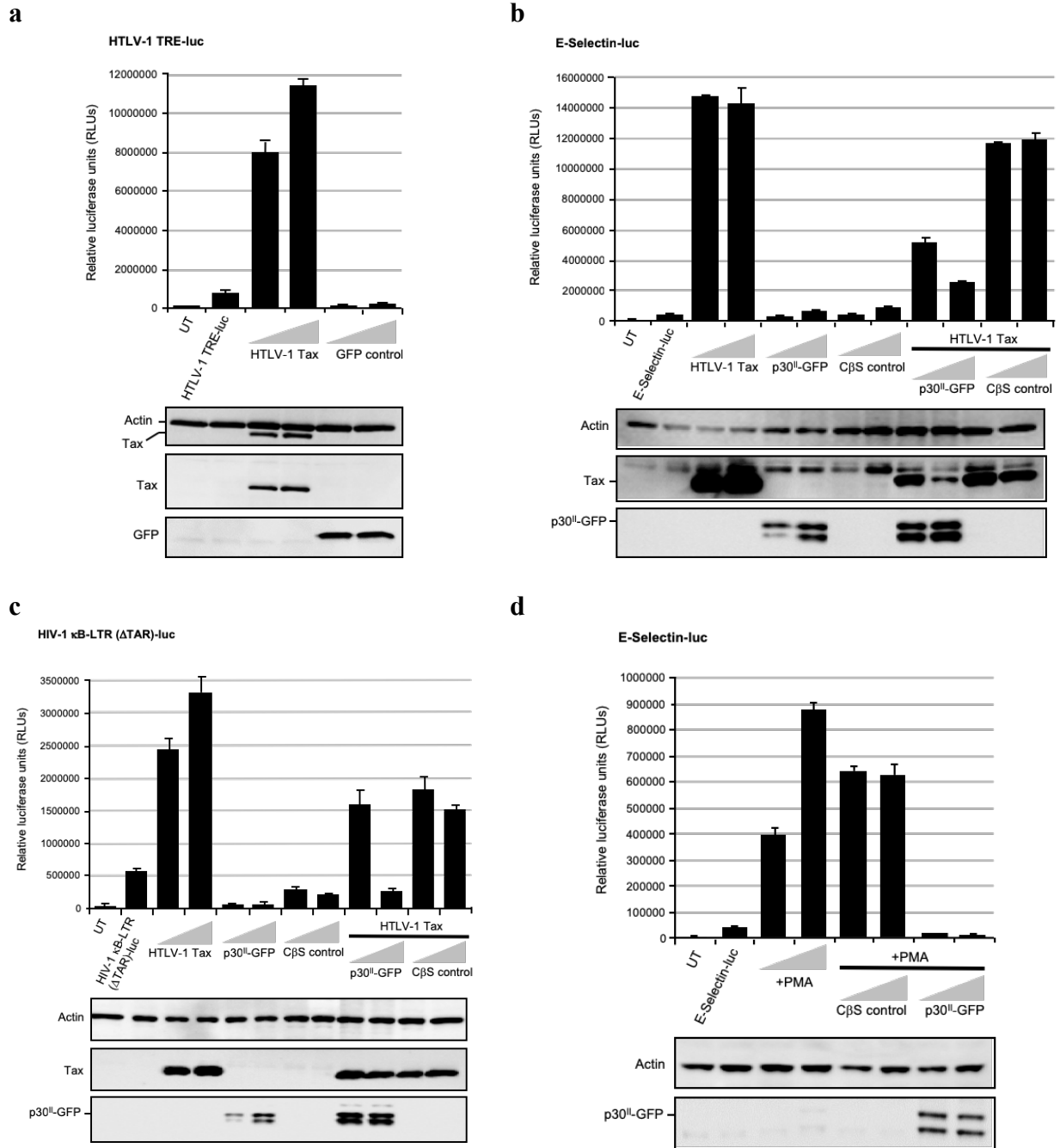
**Figure 6. The p30<sup>II</sup> protein inhibits p53-K120 acetylation and represses Stathmin/OP18 expression.** **a.** The p53 protein forms co-repressor complexes with mSin2a and HDAC1 and transcriptionally represses stathmin gene promoter (Murphy et al, 1999). **b.** p30<sup>II</sup> inhibits the K120-acetylation of p53 induced by oncogenic expression of c-Myc. **c and d.** The p30<sup>II</sup> protein inhibits Stathmin expression which results in reduced levels of p65<sup>RelA</sup> in cells expressing the viral transactivator Tax.

## 2.2 HTLV-1 p30<sup>II</sup> suppresses Tax-induced NFκB transactivation

Rationale: Tax drives proviral gene expression and replication by inducing CREB/ATF-dependent transcription from three Tax-responsive elements (TREs) within the U3 region of the HTLV-1 5' long terminal repeat (LTR) (Zhao L and Giam C, 1992). Although NFκB pathway is tightly controlled in normal T-cells, it is constitutively activated in both HTLV-I-transformed T-cell lines and freshly isolated ATL cells (Sun SC and Yamaoka S, 2005) suggesting its critical role in mediating the development of ATL. Activation of NFκB by Tax is mediated by direct binding of Tax to the regulatory subunit of IκB kinase (IKK), NFκB essential modulator (NEMO), also known as IKKγ. This interaction results in constitutive activation of IKKα and IKKβ, degradation of the IκBs, and activation of both classical and alternative NFκB pathways (Sun SC and Ballard DW, 1999; Kafoury Y et al, 2010). Tax is also known to inhibit p53 function through NFκB signaling (Qu Z and Xiao G, 2011). Thus, by constitutive NFκB activation, Tax induces NFκB target genes expression to promote cell growth and survival as well as suppresses the expression of p53 target genes involved in DNA repair and cell cycle checkpoint regulation. Both these functions of NFκB likely contribute to the induction of T-cell transformation. Although NFκB transactivation is essential for the proliferation and survival of HTLV-1-transformed lymphocytes (Zhang H et al, 2016), the Tax induced hyperactivation of NFκB-signaling leads to DNA-damage, cytotoxicity and oxidative stress that can cause cellular senescence and apoptosis (Baydoun HH et al, 2015; Zhi H et al, 2011). Hutchison et al showed that HTLV-1 p30<sup>II</sup> inhibits Tax-induced cytotoxicity by activation of p53-regulated pro-survival signals such as TIGAR, which suppress Tax-induced oxidative stress (Hutchison et al, 2018). *We therefore hypothesize p30<sup>II</sup> influences the ability of Tax to interact with IKK signaling components and might counter the hyperactivation of NFκB-signaling by the Tax transactivator protein.*

Results and Discussion: The viral transactivator drives viral gene expression and replication by interacting with the cellular transcription factors, CREB/ATF-1, and activates 21- bp-repeat Tax-responsive elements within the U3 region of the HTLV-1 LTR (Zhao LJ and Giam CZ, 1992). 293HEK cells were co-transfected with an HTLV-1 LTR luciferase reporter plasmid (Giebler HA et al, 1997; Harrod R et al, 1998) and increasing amounts of CMV-HTLV-1 Tax or a pcDNA3.1-GFP control. The HTLV-1 Tax, GFP, and Actin proteins were detected by immunoblotting (Fig. 7a). In order to determine if p30<sup>II</sup> inhibits NFκB transactivation by Tax, a κB responsive E-Selectin gene luciferase reporter promoter construct (Hong S et al, 2007) or a ΔTAR/SP1 mutant version of the HIV-1 promoter which contains only the κB-responsive elements and three SP1-binding sites with a deletion of the U-rich trinucleotide bulge of the TAR of the HIV-1<sub>LAI</sub> promoter were transfected in 293HEK cells along with expression constructs for HTLV-1 Tax, p30<sup>II</sup>-GFP, or an empty CβS vector control (Figs. 7b and 7c). These findings indicate that p30<sup>II</sup> could significantly inhibit Tax-dependent NFκB transactivation from the E-Selectin and HIV-1 κB-LTR (ΔTAR) promoter-reporter plasmids. Phorbol 12-myristate 13-acetate (PMA), also known as 12-O-tetradecanoylphorbol 13-acetate (TPA) is a specific activator of Protein Kinase C (PKC) and of NF-κB signaling (Holden NS et al, 2008). It has been previously demonstrated that PMA, and Tax can activate the promoters of several genes by causing the transcription factor NFκB to translocate from the cytoplasm to the nucleus and bind κB sites within the promoters of responsive genes to stimulate transcription. Thus, I have shown transactivation by different concentrations of PMA in 293HEK cells (Fig. 7d). p30<sup>II</sup>-GFP also suppressed NFκB transactivation induced by stimulating the cells with PMA (Fig. 7d).

The transactivation from the E-Selectin promoter through NFκB was impaired upon using the Tax mutants, Tax-M22 and -G148V, which are defective for NFκB activation (Smith MR and

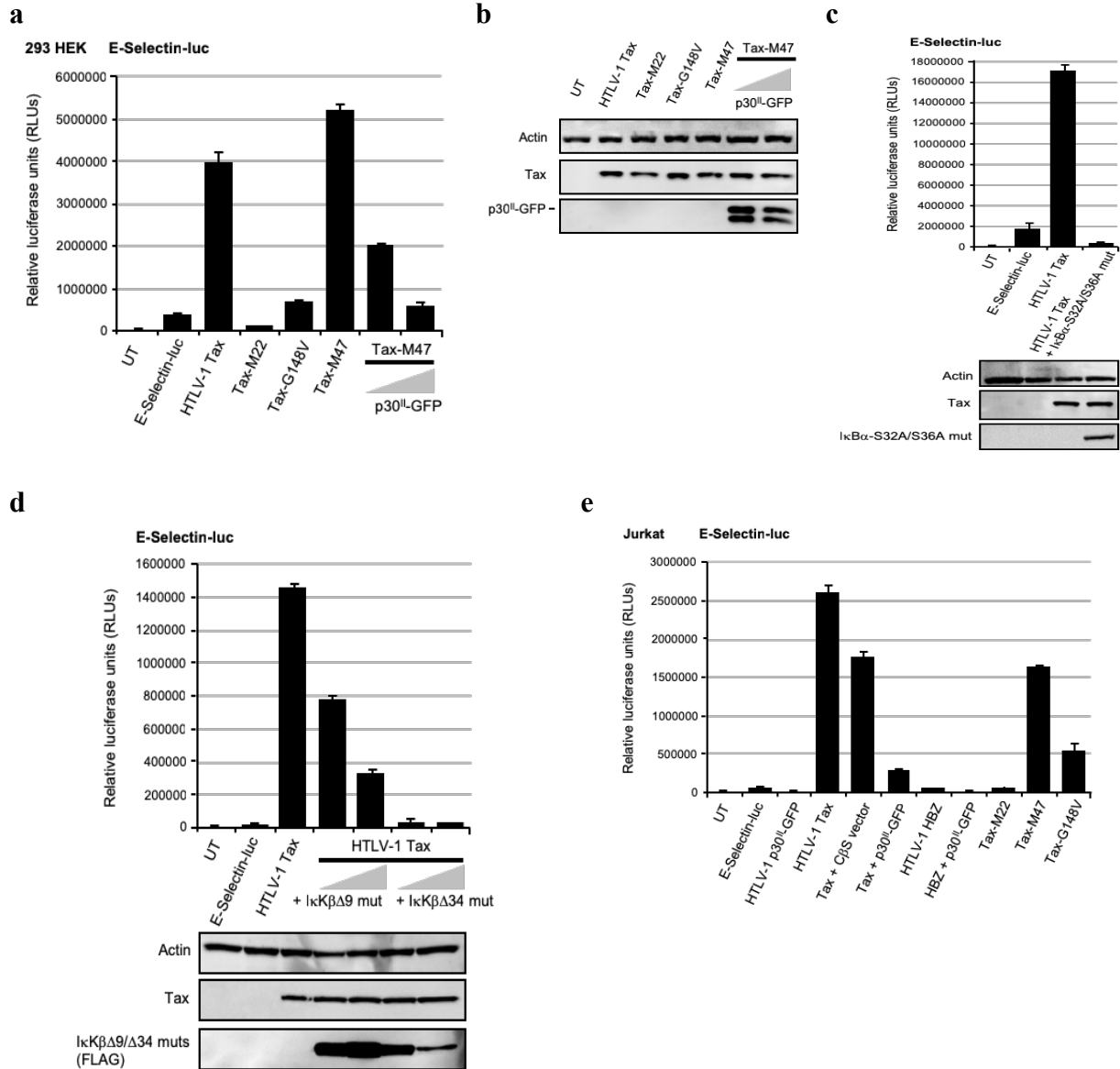


**Figure 7. HTLV-1 p30<sup>II</sup> inhibits NFκB hyper-activation by the viral oncoprotein Tax.** **a.** Transcriptional activation of an HTLV-1 LTR-luciferase reporter gene by CMV-Tax, as compared to a GFP control. **b and c.** 293 HEK cells were cotransfected with either an E-Selectin promoter-luciferase reporter plasmid or ΔTAR/SP1Mut version of an HIV-1 LTR-luciferase reporter plasmid which contains only its κB-responsive enhancer elements and increasing amounts of CMV-HTLV-1 Tax, CMV-HTLV-1 p30<sup>II</sup>-GFP, or a CβS empty vector control. **d.** 293 HEK cells were treated with PMA and NFκB transcriptional activation was observed on an *E-Selectin* promoter.

Greene WC, 1990; Yamaoka S et al, 1996), unlike the Tax-M47 mutant defective for CREB-dependent transactivation, but which supports NF $\kappa$ B-signaling (Smith MR and Greene WC, 1990) (Fig. 8a).

Furthermore, these results demonstrated that p30<sup>II</sup> inhibits NF $\kappa$ B activation by the M47 Tax mutant (Fig. 8a). Immunoblotting was performed to detect the wildtype Tax and the Tax-M22, -M47 and -G148V mutant proteins, as well as p30<sup>II</sup>-GFP (Fig. 8a). The Tax-dependent NF $\kappa$ B activation from the E-Selectin promoter was also impaired when 293HEK cells were co-transfected with the dominant-negative deletion mutants of I $\kappa$ K $\beta$  subunit: I $\kappa$ K $\beta$  $\Delta$ 9 and I $\kappa$ K $\beta$  $\Delta$ 34 (Sylla BS et al, 1998) (Fig. 8c), or the “super repressor” S32A/S36A mutant of I $\kappa$ B $\alpha$  which is defective for serine-phosphorylation and proteasome-mediated degradation (DiDonato J et al, 1996) (Fig. 8b).

Since HTLV-1 infects CD4<sup>+</sup> T-cells, I have further shown Tax induced transactivation of NF $\kappa$ B, and the repression by p30<sup>II</sup> in Jurkat T-cells (Fig. 8d). The HTLV-1 bZIP factor (HBZ), which is encoded by an mRNA of the opposite polarity of the viral genomic RNA, is involved in both T-cell proliferation and suppression of Tax-mediated viral gene transcription (Clerc I et al, 2008), suggesting that HBZ cooperates closely with Tax. It has been shown that HBZ specifically suppresses NF $\kappa$ B-driven transcription mediated by p65 (the classical pathway), without inhibiting the alternative NF $\kappa$ B signaling pathway (Satou and Matsuoka, 2012; Zhao and Matsuoka, 2012). Furthermore, the selective suppression of the classical NF $\kappa$ B pathway by HBZ renders the alternative NF $\kappa$ B pathway predominant after activation of NF $\kappa$ B by Tax or other stimuli (Zhao T et al, 2009). My results demonstrate that HBZ is not able to significantly activate NF $\kappa$ B transactivation on E. Selectin promoter and that p30<sup>II</sup> suppresses the minimal activity that HBZ has on this promoter (Fig. 8d). Consistent with figure 8a, NF $\kappa$ B transactivation in Jurkat T-cells



**Figure 8. Tax-G148V mutant and dominant-negative mutants of IκBα or IκKβ inhibit Tax-induced NFκB transactivation.** **a.** Luciferase activity was measured on an E-Selectin promoter-luciferase reporter in 293 HEK cells that were co-transfected with the luciferase reporter construct and either CMV-HTLV-1 Tax (wild-type) or the Tax mutants, M22, M47, or G148V. The cells were also co-transfected with the M47 Tax mutant in the presence of increasing amounts of CMV-HTLV-1 p30<sup>II</sup>-GFP. **b.** Immunoblots to detect protein expression in “8a”. **c and d.** The NFκB-dependent transactivation from the *E-Selectin* gene promoter by HTLV-1 Tax was demonstrated by co-transfecting the cells with CMV-HTLV-1 Tax in the presence of dominant-negative mutants of the IκKβ catalytic subunit: IκKβΔ9 and IκKβΔ34 (Sylla et al, 1998), or pRc-IκBα-S32A/S36A, a “super repressor” mutant of the IκBα inhibitor, defective for Ser34/Ser36-phosphorylation and degradation (DiDonato et al, 1996). **e.** Jurkat T-lymphocytes were cotransfected with an *E-Selectin* promoter-luciferase reporter plasmid and either CMV-HTLV-1 Tax (wild-type) or the Tax mutants, M22, M47, or G148V and relative luciferase values were determined.

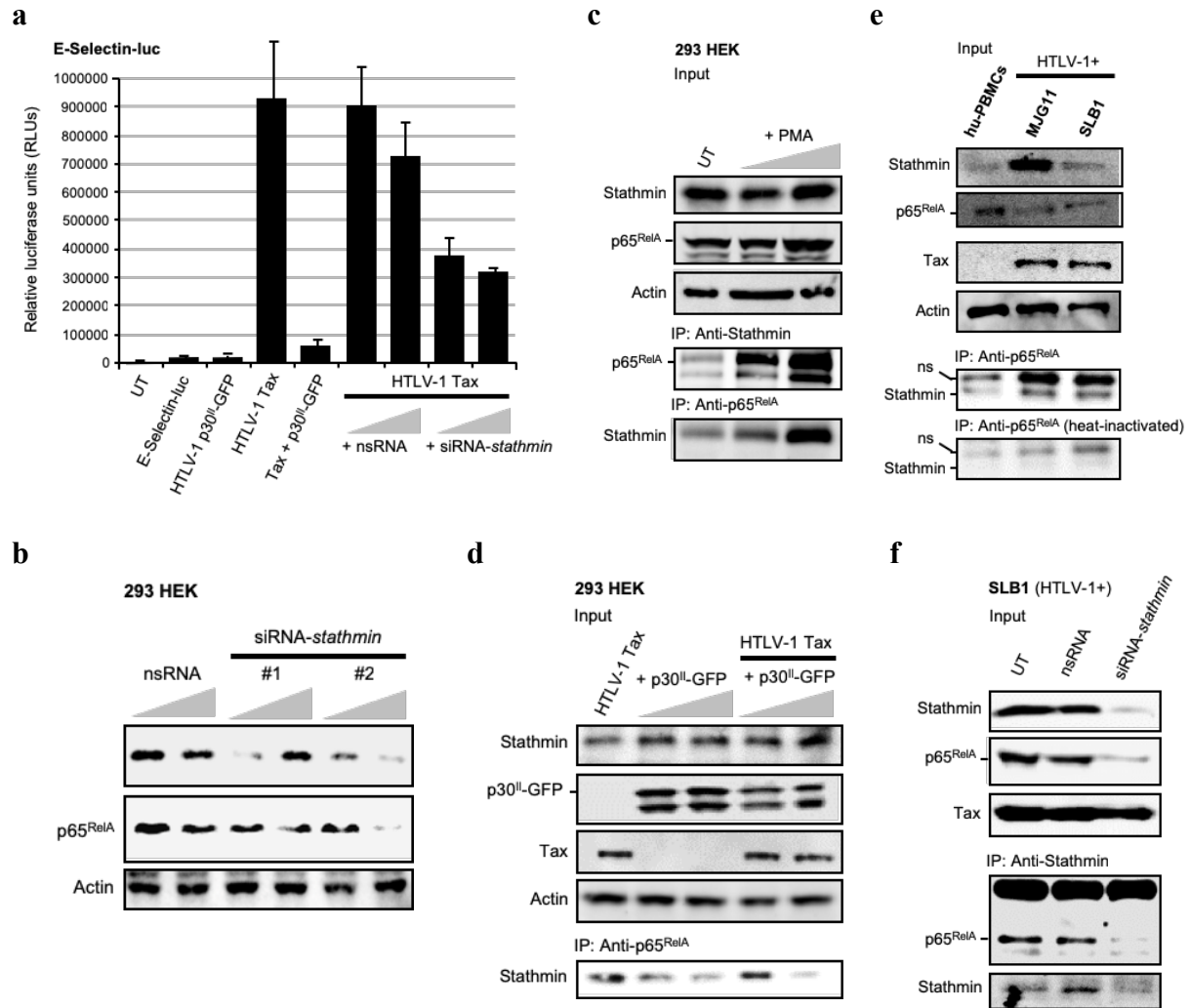
demonstrated that Tax mutants M22 and Tax-G148V were impaired, whereas Tax-M47 induced NFκB-dependent transcriptional activation at levels comparable to wildtype Tax (Fig. 8d).

### **2.3 Stathmin and p65<sup>RelA</sup> interact in HTLV-1 transformed T-cell lines and knockdown of Stathmin destabilizes NFκB/p65<sup>RelA</sup> subunit**

Rationale: Lu et al demonstrated a correlation between stathmin and NFκB pathway in pancreatic cancer. In pancreatic cancer environment, it was previously demonstrated that microtubule disruption contributed to an enhanced chronic hypoxia and microtubule destabilization induced a significant Hypoxia-inducible factor 1-alpha (HIF1-α) up-regulation through an NFκB-dependent pathway (Jung YJ et al, 2003). Lu et al proposed that it is possible that stathmin played its oncogenic role also by utilizing a potential NFκB mediated pathway in pancreatic cancer. They found that stathmin binds to and stabilizes the p65<sup>RelA</sup> subunit of NFκB and also confirmed that p65<sup>RelA</sup> was downregulated when stathmin was knocked-down suggesting a novel oncogenic role of stathmin in aggressive pancreatic cancer (Lu Y et al, 2014). Lu et al also found that silencing of Stathmin inhibited the pancreatic cancer cell proliferation *in vitro* and *in vivo* (Lu Y et al, 2014). Given this novel oncogenic role of Stathmin, we sought to determine the effect of depleting Stathmin upon Tax-induced NFκB transactivation using the E. Selectin luciferase construct (Fig. 9b). The NFκB pathway plays a crucial role in HTLV-1 associated oncogenesis (Hiscott J et al, 2001). *We hypothesize that there is an underlying connection between Stathmin and NFκB/p65<sup>RelA</sup> where Stathmin participates in Tax-induced NFκB transactivation through an interaction with p65<sup>RelA</sup> subunit.*

Results and Discussion: In order to determine whether stathmin knockdown can affect Tax-induced NFκB transactivation, 293HEK cells were co-transfected with siRNA oligonucleotides targeted against stathmin transcripts (siRNA-stathmin) or a non-specific RNA (nsRNA) as





**Figure 9. Stathmin interacts with the NF $\kappa$ B p65<sup>RelA</sup> subunit in HTLV-1-transformed ATLL cells and Stathmin knockdown inhibits Tax-induced NF $\kappa$ B transactivation.** **a.** 293 cells were cotransfected with an *E-Selectin* luciferase reporter plasmid and expression constructs for wt Tax or p30<sup>II</sup>-GFP. Some samples were transfected with wt Tax and increasing amounts of non-specific RNA (nsRNA) negative control or an siRNA targeted against *stathmin* transcripts (siRNA-*stathmin*). Relative luciferase values were determined. **b.** Immunoblots to detect siRNA-knockdown of Stathmin protein expression. Stathmin depletion destabilized p65<sup>RelA</sup> and Actin is shown as a control. **c.** Co-immunoprecipitation of Stathmin and p65<sup>RelA</sup> in 293 HEK cells stimulated with increasing concentrations of phorbol 12-myristate 13-acetate (PMA). **d.** Effects of HTLV-1 p30<sup>II</sup> upon p65<sup>RelA</sup>-Stathmin interactions in Tax-expressing 293 cells. Input blots depicting protein expression for Tax and p30<sup>II</sup>-GFP are shown. **e.** Co-immunoprecipitation for Stathmin and p65<sup>RelA</sup> in cultured activated hu-PBMCs and in the HTLV-1+ve MJG11 and SLB1 T-cell lines. Heat-denatured Anti-p65<sup>RelA</sup> antibody was included as a negative control (lower panel). **f.** Knockdown of Stathmin expression in SLB1 lymphoblasts using siRNA-*stathmin* destabilized p65<sup>RelA</sup> and Stathmin-p65<sup>RelA</sup> complexes were significantly reduced.

negative control. We used two different siRNAstathmin oligonucleotides (#1 and #2) and observed that they both significantly inhibited Stathmin expression and also destabilized the p65<sup>RelA</sup> protein (Figs. 9a and 9b). Relative actin levels are shown by immunoblotting (Fig. 9b). Next, we observed that targeted knockdown of Stathmin could also inhibit Tax-mediated NFκB transactivation (Fig. 9a). Since the NFκB signaling pathway is widely associated with the regulation of cell survival, inflammation, and cell proliferation, it was logical to test and verify whether stathmin could play its oncogenic role by activating an NFκB pathway. In order to assess this, we performed western blots and co-immunoprecipitation assays in PMA activated 293 HEK cells which activates NFκB signaling in these cells. We observed an increased expression of the p65<sup>RelA</sup> protein compared to the relative input levels of Actin (Fig. 9c). Co-immunoprecipitation of Stathmin/Op-18 and p65<sup>RelA</sup> complexes was seen using Protein-G-agarose and AntiStathmin/Op-18 or Anti-p65<sup>RelA</sup> antibodies in PMA-treated cells (Fig. 9c). Since we earlier saw that HTLV-1 p30<sup>II</sup> suppressed Stathmin (Fig. 6c and 6d), we wanted to see the effect of p30<sup>II</sup> on Stathmin-p65<sup>RelA</sup> complexes in cells with Tax-induced NFκB activation (Fig. 9d). We used Anti-p65<sup>RelA</sup> antibody to coimmunoprecipitate p65<sup>RelA</sup> protein with Stathmin and saw that p30<sup>II</sup>-GFP, either alone or in combination with Tax, inhibited their co-immunoprecipitation (Fig. 9d). The input levels of Tax, p30<sup>II</sup>-GFP, Stathmin, and Actin are shown (Fig. 9d).

Next, we used ATLL tumor cell-lines that express high levels of the Tax oncoprotein to study the interaction between p65<sup>RelA</sup> and Stathmin using an Anti-p65<sup>RelA</sup> antibody and extracts prepared from the HTLV-1-transformed lymphoma T-cell-lines, MJG11 and SLB1, and cultured human PBMCs as control cells (Fig. 9e). The results in figure 9e demonstrate that the NFκB/p65<sup>RelA</sup> subunit was coimmunoprecipitated in Stathmin-containing complexes indicating a stable interaction between p65<sup>RelA</sup> and Stathmin in HTLV-1-transformed T-cell lines which was

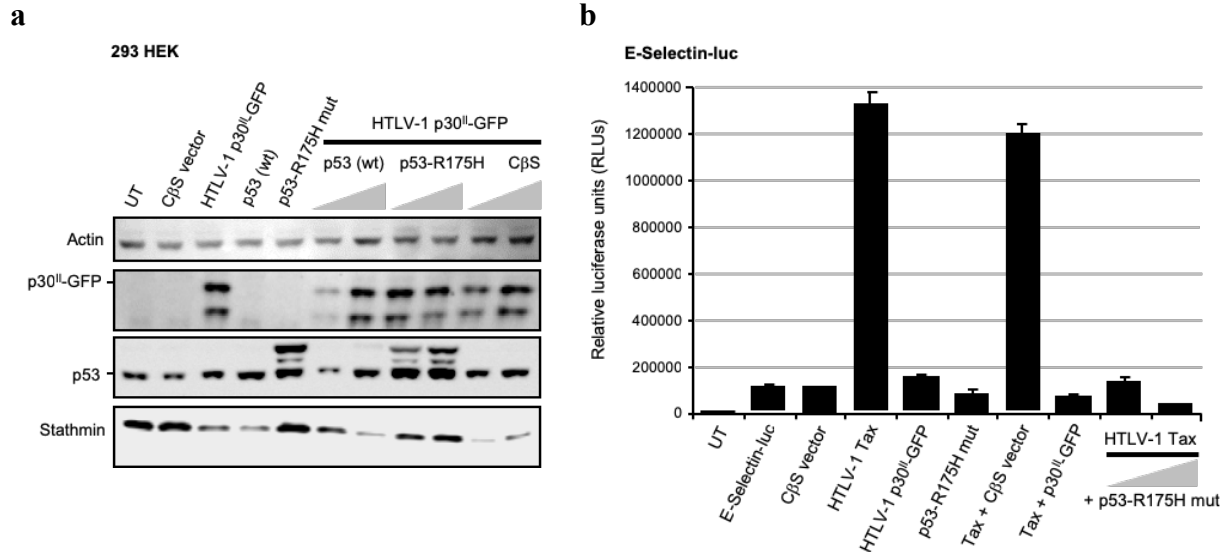
significantly higher than the control lane with hu-PBMCs (Fig. 9e). Heat-inactivated monoclonal Anti-p65<sup>RelA</sup> antibody was used as a negative control in co-immunoprecipitation reactions (Fig. 9e, bottom panel). Further, the targeted knockdown of Stathmin using si-RNA oligonucleotide (#2) inhibited the formation of p65<sup>RelA</sup>-Stathmin immune-complexes in the SLB1 HTLV-1-transformed lymphoma T-cell-line, as compared to nsRNA negative control (Fig. 9f).

It has been reported in other studies that Stathmin/Op-18 binds to the NFκB subunit p65<sup>RelA</sup> and functions as a cofactor by stabilizing the p65<sup>RelA</sup> in aggressive pancreatic cancers (Lu Y et al, 2014). The coimmunoprecipitation of Stathmin and p65<sup>RelA</sup> in stable complexes (Figs. 9c, 9d, 9e and 9f) and the inhibition of these immune-complexes either in the presence of HTLV-1 p30<sup>II</sup> (Fig. 9d) or upon targeted knockdown of Stathmin (Fig. 9f) clearly demonstrate a possible role of Stathmin in stabilizing NFκB/p65<sup>RelA</sup> subunit and subsequent activation of NFκB pathway in HTLV-1 transformed T-cells.

## **2.4 HTLV-1 p30<sup>II</sup> represses Stathmin through a p53-dependent mechanism**

Rationale: Stathmin is a gene negatively regulated by p53 tumor suppressor, being transcriptionally repressed by p53/mSin3a/HDAC1 complexes (Murphy M et al, 1999). Additionally, our lab has demonstrated that HTLV-1 latency protein p30<sup>II</sup> activates p53 (Romeo M et al, 2018). In the previous figures I was able to show that the p30<sup>II</sup> protein inhibits Stathmin (Figs. 6c and 6d) and also destabilizes Stathmin-p65<sup>RelA</sup> complexes (Fig. 9d). Unlike most other cancers, the p53 tumor suppressor is rarely mutated in HTLV-1 associated oncogenesis (Zane L et al, 2012) alluding to a possible role of p53-regulated mechanism in HTLV-1 pathogenesis.

***Together these findings led us to explore whether using a p53-dominant negative transcriptional mutant will affect the ability of p30<sup>II</sup> to inhibit Stathmin.***



**Figure 10. Coexpression of the dominant-negative R175H DNA-binding mutant of p53 prevents the suppression of Stathmin expression by HTLV-1 p30<sup>II</sup>.** **a.** 293 HEK cells were cotransfected with various combinations of HTLV-1 p30<sup>II</sup>-GFP, pCEP4-wildtype-p53, pCEP4-p53-R175H mutant, or an empty CβS vector control plasmid, and the expression of p30<sup>II</sup>-GFP, wildtype and R175H mutant p53, and Stathmin proteins was detected by immunoblotting. Actin levels are shown as a control for protein loading. **b.** To determine the effects of the dominant-negative p53-R175H mutant upon Tax-dependent NFκB transcriptional activation, 293 HEK cells were cotransfected with an *E-Selectin* luciferase reporter plasmid and expression constructs for HTLV-1 Tax, p30<sup>II</sup>-GFP, the p53-R175H DNA-binding mutant, or an empty CβS vector control. Relative luciferase values were measured by normalizing to total cellular protein.

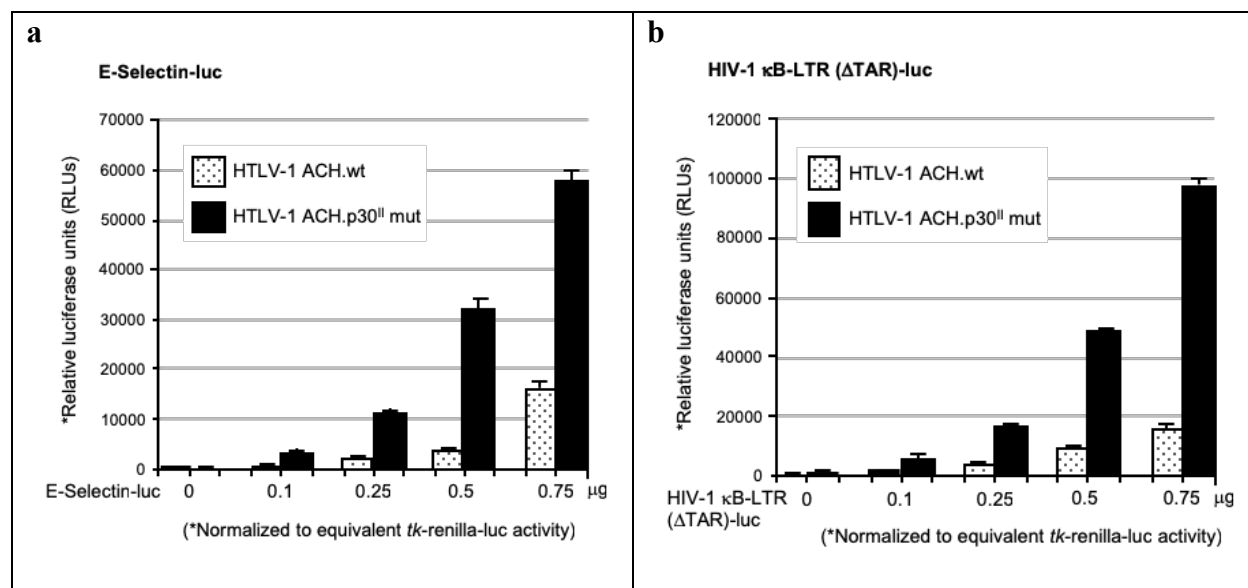
Results and Discussion: A dominant-negative DNA binding-defective mutant of p53; p53-R175H (Hermeking H et al, 1997) was used to assess the effect of p30<sup>II</sup> on Stathmin expression. 293 HEK cells were co-transfected with various expression constructs for HTLV-1 p30<sup>II</sup>-GFP, wildtype p53, p53 mutant p53-R175H, or an empty CβS vector control. The expression of the Stathmin, p30<sup>II</sup>-GFP, p53 and Actin proteins was detected by immunoblotting (Fig. 10a). We observed that as compared to the untransfected control or the CβS vector control, p30<sup>II</sup>-GFP significantly inhibited the expression of Stathmin (Figs. 10a and 10b). Since p53 is a negative regulator of Stathmin and transcriptionally represses its expression, we expected that wildtype p53

overexpression might also suppress Stathmin protein. Indeed, as expected, the overexpression of wildtype p53 also inhibited Stathmin protein expression (Figs. 10a and 10b). Moreover, the dominant-negative DNA-binding mutant of p53, p53-R175H, led to increased levels of Stathmin and also countered the ability of p30<sup>II</sup>-GFP to repress Stathmin (Figs. 10a and 10b). To study the effect of p53-R175H on Tax-induced NFκB transactivation, 293 HEK cells were co-transfected with E-selecting luciferase construct and either CβS vector control, HTLV-1 Tax, p30<sup>II</sup>-GFP or p53-R175H expression constructs (Fig. 10b). It was found that the p53-R175H mutant inhibited Tax-induced NFκB transactivation from the E-Selectin promoter (Fig. 10b). This could be explained by the possibility that the p53-R175H mutant binds to and interferes with p65<sup>RelA</sup> transcription complexes (Jeong SJ et al, 2004; Pise-Masison CA et al, 2000). Another possibility could be that the p53R175H mutant might hinder Tax from interacting with the IκK-γ subunit of the IκK complex (Yamaoka S et al, 1998; Pise-Masison CA, 1998). *These results confirm that the HTLV-1 latency protein p30<sup>II</sup> represses Stathmin/Op18 in a p53-dependent manner.*

## **2.5 The role of p30<sup>II</sup> in Tax-induced NFκB-signaling in context of the full-length HTLV-1 provirus**

Rationale: We have demonstrated that HTLV-1 p30<sup>II</sup> suppresses Tax-induced NFκB transactivation in 293 HEK cells transfected with expression constructs for HTLV-1 Tax, HTLV-1 p30<sup>II</sup> and various NFκB-transactivation mutants of Tax (Figs. 7b, 7c, 7d, 8a, 8d). In order to put this finding in perspective of the full-length HTLV-1 provirus with all its regulatory and accessory proteins we used HT1080 clones containing the infectious HTLV-1 ACH.wildtype or ACH.p30<sup>II</sup> mutant provirus, defective for p30<sup>II</sup> production (Kimata JT et al, 1994; Robek MD et al, 1998; Bartoe JT et al, 2000; Romeo M et al, 2018; Hutchison et al, 2018). *We sought to extend our*

*findings that HTLV-1 p30<sup>II</sup> represses Tax-induced NFκB transactivation in context of full-length HTLV-1 provirus.*



**Figure 11. HTLV-1 p30<sup>II</sup> suppresses NFκB transactivation in ACH.wildtype proviral clones when compared to ACH.p30<sup>II</sup> mutant.** **a.** Infectious HT1080/HTLV-1 ACH.wildtype (wt) or ACH.p30<sup>II</sup> mutant proviral clones (defective for p30<sup>II</sup> production) were cotransfected with a fixed concentration (0.25μg) of *tk*-renilla-luciferase plasmid and increasing amounts of an *E-Selectin* promoter-firefly-luciferase. Dual luciferase assays were carried out and the relative NFκB-dependent firefly-luciferase activities were quantified and normalized for equivalent renilla-luciferase levels. **b.** HIV-1 κB-LTR (ΔTAR)-firefly-luciferase reporter plasmid was used in increasing amounts against a fixed concentration of *tk*-renilla-luciferase and dual luciferase assay was carried out in the same way as (11a).

Results and Discussion: HTLV-1 p30<sup>II</sup> protein suppresses the expression of Stathmin and destabilizes p65<sup>RelA</sup> and inhibits Tax-induced NFκB transactivation (Figs. 6, 7, 8 and 9). So far, we conducted our experiments in 293 HEK cells, hence to observe the effect of p30<sup>II</sup> in context of full-length HTLV-1 provirus we used infectious HTLV-1 ACH.wildtype or ACH.p30<sup>II</sup> mutant (defective for p30<sup>II</sup> production) clones. Dual luciferase assays were performed in ACH clones which were co-transfected with a *tk*-renilla-luciferase plasmid and increasing amounts of a κB-

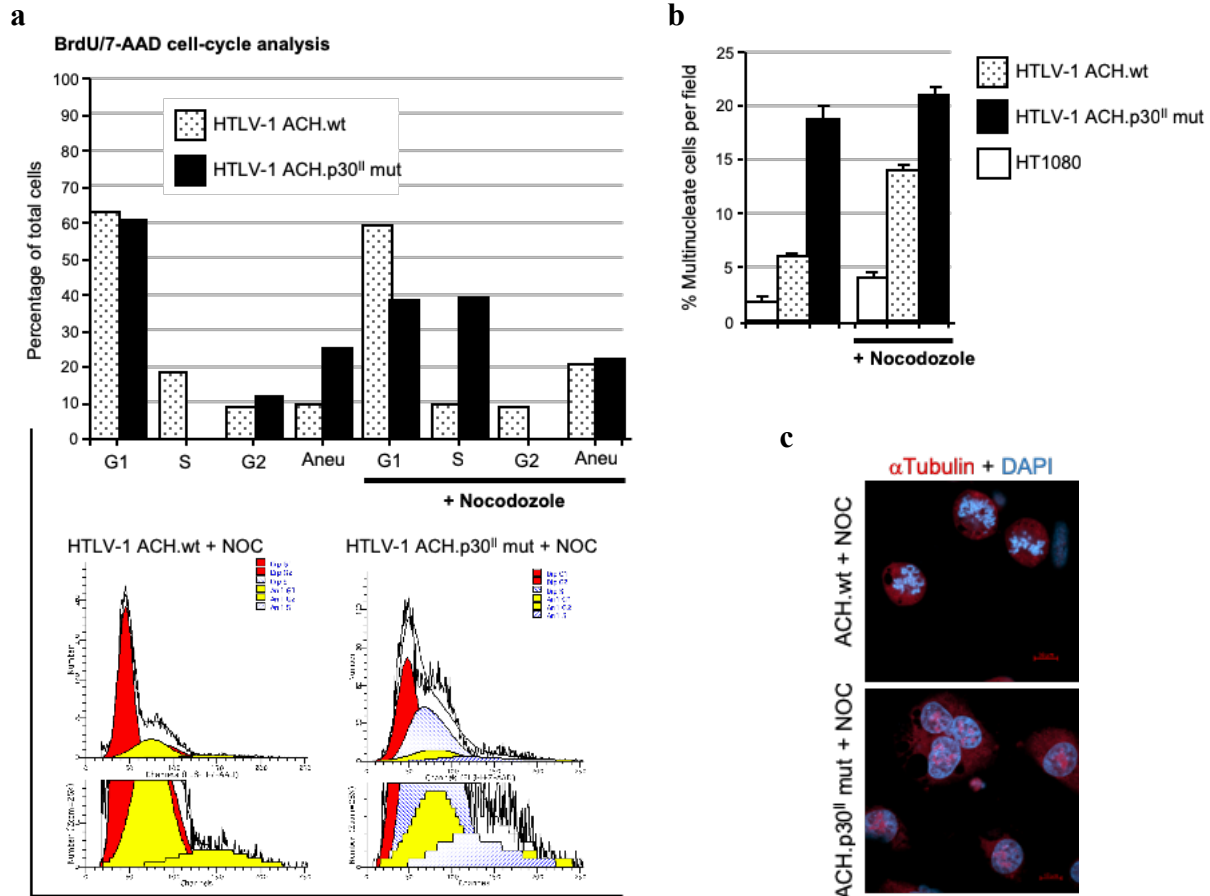
responsive E-Selectin-firefly luciferase (Fig. 11a) or HIV-1  $\kappa$ B-LTR ( $\Delta$ TAR)- firefly-luciferase reporter plasmid (Fig. 11b). The relative values of  $\kappa$ B-responsive firefly-luciferase activities were normalized to equivalent renilla luciferase levels which revealed that NF $\kappa$ B-dependent transactivation was greater in the HT1080 clones expressing the HTLV-1 ACH.p30<sup>II</sup> mutant provirus, compared to wildtype ACH. It is however to be noted that the mutation in ACH.p30<sup>II</sup> provirus also results in an in-frame insertion of eight amino acids (YLEEESRG) after the activation domain of the antisense HBZ protein (Robek MD et al, 1998; Clerc I et al, 2008) this insertion however is not likely to disrupt HBZ. Nevertheless, the possibility that the ACH.p30<sup>II</sup> mutant provirus could also impair HBZ functions cannot be ruled out. In fact, previous studies have shown that HBZ also inhibits Tax-induced NF $\kappa$ B transactivation (Zhi et al, 2011). Hence, it can also be possible that the inactivation of both p30<sup>II</sup> and HBZ could produce a combined effect upon NF $\kappa$ B-signaling. *Nonetheless, these findings are in consensus with our previous results that the viral latency protein p30<sup>II</sup> dampens Tax-induced NF $\kappa$ B-signaling.*

## **2.6 HTLV-1 p30<sup>II</sup> suppresses Tax-induced microtubule (MT) destabilization and genomic instability**

Rationale - Tax has a weak oncogenic activity in ATL cells but the genomic instability caused by Tax plays a key role in the development of ATL (Baydoun H et al, 2012). Clastogenic DNA damage including structural and numerical chromosomal rearrangements are frequently seen in HTLV-1 transformed T-cells and Tax expressing cells (Marriott S et al, 2002). Several studies have pointed to the role of NF $\kappa$ B signaling in genomic instability by different mechanisms such as double-strand DNA breaks, disruption of centrosome assembly, and interference with cell-cycle, to name a few. Tax-induced constitutive activation of NF $\kappa$ B signaling increases the expression of iNOS and NO production causing dsDNA-breaks in HTLV-1-infected cells

(Baydoun et al, 2015). Chaib-Mezrag et al showed that Tax induces DDSBs through activation of NF $\kappa$ B pathway and by stimulating intracellular NOS (Chaib-Mezrag H et al, 2014). The direct binding of  $\kappa$ B-responsive elements in the plk4 gene promoter (Polo-like kinase 4) regulate the expression of Polo-like kinase 4 (PLK4) and may disrupt the assembly of centrosomes during the cell cycle (Ledoux AC et al, 2013). Stathmin interacts directly with tubulin and can destabilize microtubules by sequestering tubulin heterodimers leading to a decrease in amount of free tubulin available for microtubule assembly and hence promoting catastrophes (Charbaut E et al, 2001). This microtubule disruption induced by stathmin may lead to formation of abnormal mitotic spindle and inaccurate chromosomal segregation and is considered to be aneugenic since such defects contribute to aneuploidy. Previous studies have shown that stathmin plays an oncogenic role in promoting cancer cell proliferation, differentiation, migration and invasion (Lu Y et al, 2014). Stathmin is upregulated in several cancers and overexpression of wild-type Op18 results in aneugenic activities, leading to aberrant mitosis, polyploidy, and chromosome loss (Holmfeldt et al, 2006). In addition to structural chromosomal aberrations, majority of cancers typically present numerical chromosomal changes including loss or gain of entire chromosomes (aneuploidy) owing to unequal chromosomal division. ATL cells have highly lobulated characteristic “flower-shaped” nuclei (Johnson JM et al, 2001) suggesting altered MT dynamics. Our results confirm the interaction between p65<sup>RelA</sup> and Stathmin (Figs. 9c-f), which also implies that this interaction could alter MT dynamics controlled by Stathmin and lead to genomic instability in HTLV-1 associated leukemogenesis. *We propose that the interactions between p65<sup>RelA</sup> and Stathmin due to Tax-induced NF $\kappa$ B signaling leads to MT- and genomic-instability which can be suppressed by HTLV-1 p30<sup>II</sup> or inhibitors of NF $\kappa$ B transcriptional signaling.*



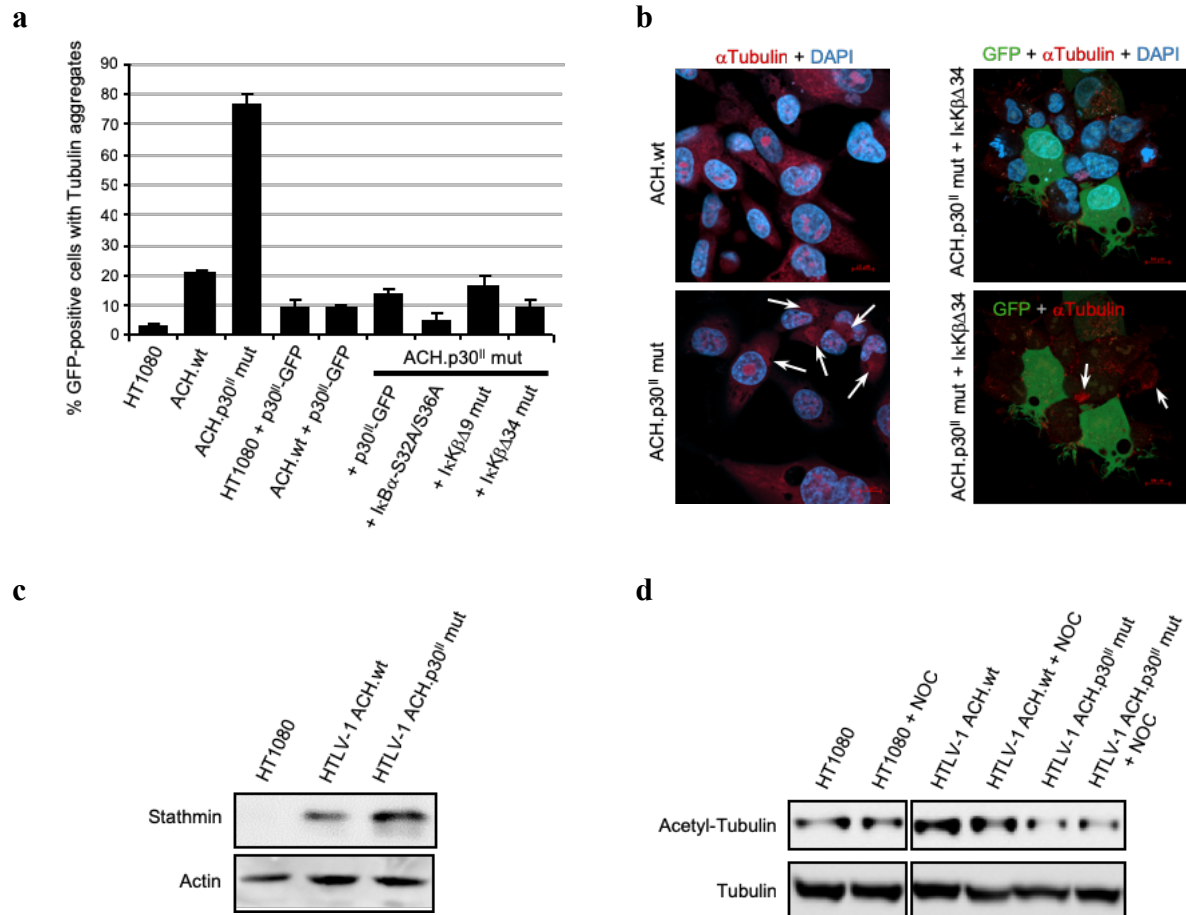


**Figure 12. HTLV-1 p30<sup>II</sup> protein inhibits Tax-induced multinucleation in the ACH proviral clones.** **a.** BrdU labeling was done on nocodazole treated HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> mutant proviral clones. Flow cytometry to determine the relative percentages of diploid cells in the G1, S, and G2-phases, as well as the total percentage of aneuploid cells using the ModFit LT 3.0 cell-cycle algorithm. **b. and c.** Immunostaining on HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> mutant proviral clones, treated with or without nocodazole. Anti-Tubulin primary antibody and a rhodamine red secondary antibody were used along with DAPI nuclear stain. Percent multinucleate cells per field were determined by in triplicate visual fields using confocal immunofluorescence-microscopy.

Results and Discussion: HTLV-1 Tax plays a crucial role in cell cycle control, Tax can prevent apoptosis and promote cell proliferation by disrupting cell cycle check points in virus infected cells. Tax interacts with cell cycle components and often results in dysregulation causing cellular abnormalities, aneuploidy and immortalization of T-cells which ultimately lead to ATL (Curren R

et al, 2012). Stathmin has been shown to compromise the fidelity of spindle assembly, increase chromosomal instability and is considered to be aneugenic (Holmfeldt et al, 2006). Stathmin can interact with p65<sup>RelA</sup> and possibly affect MT dynamics and promote genomic instability in HTLV-1 infected cells. We addressed this possibility by performing flow-cytometry for cell-cycle analysis in the infectious HTLV-1 ACH.wildtype or ACH.p30<sup>II</sup> mutant (defective for p30<sup>II</sup> production) clones labeled with bromodeoxyuridine (BrdU), in the absence or presence of nocodazole-treatment (Fig. 12a). We used the ModFit LT 3.0 algorithm to calculate the relative percentages of cells in the G1, S, or G2-phases, or those with aneuploidy. We discovered that the ACH.p30<sup>II</sup> mutant proviral clones exhibited significant aneuploidy in the absence of nocodazole and even bypassed nocodazole-induced metaphase-phase arrest showing an increased percentage of nocodazole-treated cells in the S-phase (Fig. 12a). Next, we treated the HTLV-1 ACH.wt and ACH.p30<sup>II</sup> proviral clones with nocodazole and then stained with DAPI and a primary antibody against Alpha-Tubulin and a fluorescence-conjugated secondary antibody. The results were then analyzed by immunofluorescence-confocal microscopy and the number of multinucleate cells per field were quantified in triplicates. This experiment further confirmed our findings that the ACH.p30<sup>II</sup> mutant clones (lacking a functional p30<sup>II</sup> protein) exhibit higher percentage of multinucleate cells, even in the absence of nocodazole-treatment when compared to ACH.wt clones (Figs. 12b and 12c) where the p30<sup>II</sup> protein possibly represses Stathmin and NFκB dependent transactivation. We expected ACH.wt clones to have repression of Stathmin by p30<sup>II</sup> protein, compared to ACH.p30<sup>II</sup> mutant clones (lacking p30<sup>II</sup>) and indeed that's what we observed by immunoblot analysis (Fig. 12d). Thus, we next wanted to determine if the HTLV-1 ACH.p30<sup>II</sup> mutant exhibits more MT-instability than the ACH.wt clones.

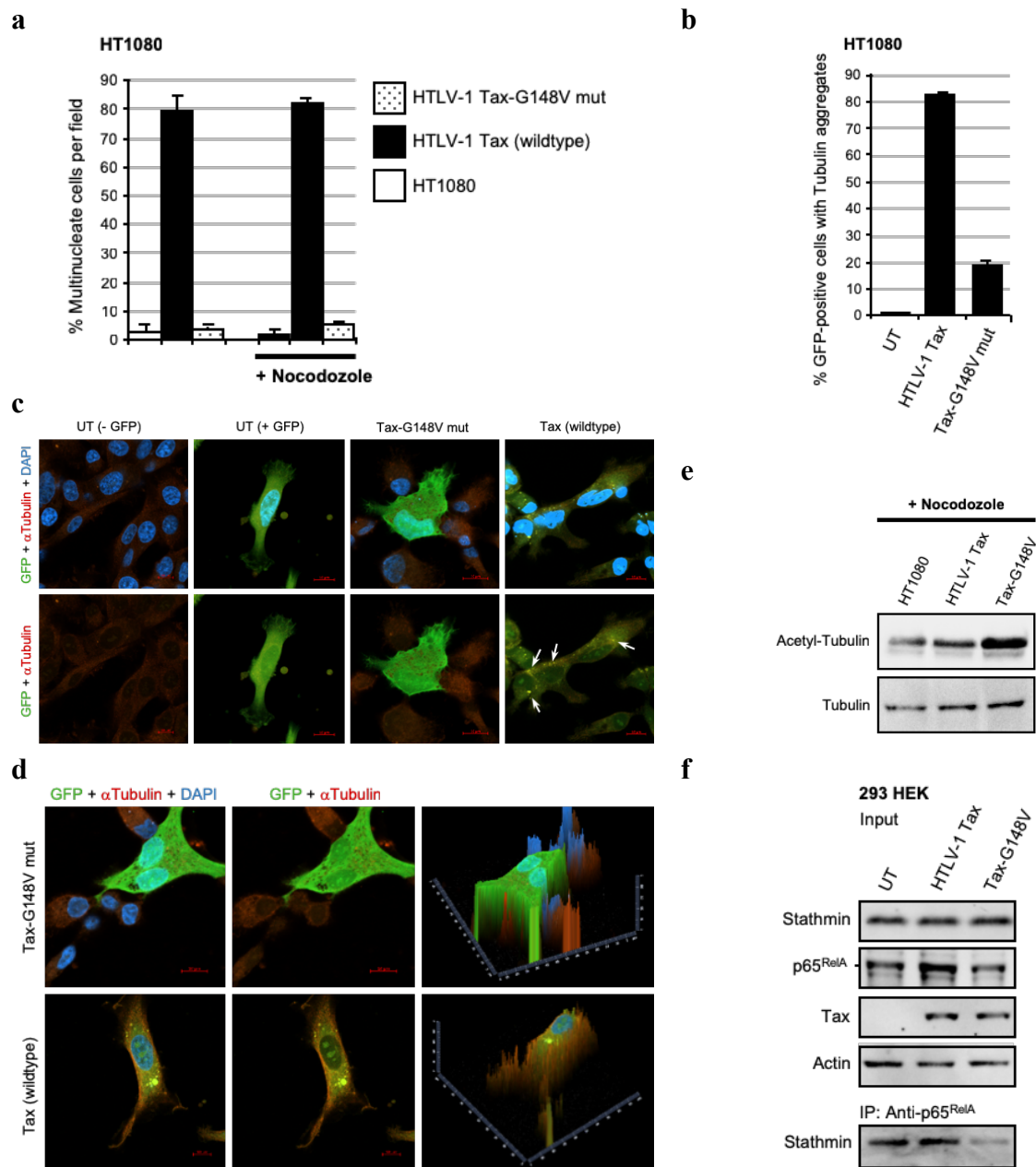
In order to assess this, the parental HT1080 cells, or HT1080/HTLV-1 ACH.wt or ACH.p30<sup>II</sup> mutant proviral clones, were transfected with p30<sup>II</sup>-GFP, or pcDNA3.1-GFP and inhibitors of



**Figure 13. HTLV-1 p30<sup>II</sup> protein inhibits Tax-induced MT-destabilization in the ACH proviral clones.** **a. and b.** Immunostaining using Anti-Tubulin primary antibody and rhodamine-red-conjugated secondary antibody (red) and DAPI-nuclear stain (blue) were performed on HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> mutant proviral clones transfected with HTLV-1 p30<sup>II</sup>-GFP, or dominant-negative mutants: IκBα-S32A/S36A, IκKβΔ9, or IκKβΔ34, and co-transfected with pcDNA3.1-GFP. As a control, HT1080 cells were transfected with pcDNA3.1-GFP. The relative percentages of GFP-positive cells with cytoplasmic tubulin aggregates (indicated by arrows in 13.b) were quantified in-triplicate by confocal immunofluorescence imaging. **c.** Immunoblots to detect Stathmin protein expression in the HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> mutant proviral clones, compared to control HT1080 cell-line. Actin protein was detected for loading control. **d.** Immunoblots to detect levels of acetylated Alpha-Tubulin in HT1080 cells or HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> mutant proviral clones, ± nocodazole-treatment and bottom panel shows total Alpha-Tubulin as control.

NF $\kappa$ B - various expression constructs for the I $\kappa$ B $\alpha$ -S32A/S36A (DiDonato J et al, 1996), I $\kappa$ K $\beta$  $\Delta$ 9, or I $\kappa$ K $\beta$  $\Delta$ 34 mutants (Sylla BS et al, 1998).

The relative percentages of transfected GFP-positive cells containing cytoplasmic tubulin aggregates were quantified using immunofluorescence-confocal microscopy. Upon quantification, the ACH.p30<sup>II</sup> mutant clones exhibited much higher number of cytoplasmic tubulin aggregates relative to the ACH.wt clones (Figs. 13a and b; arrows in Figs. 13b) indicating aberrant MT dynamics in ACH.p30<sup>II</sup> mutant clones which can be attributed to Stathmin overexpression in the absence of p30<sup>II</sup> protein. I then transfected ACH.p30<sup>II</sup> mutant clones with either p30<sup>II</sup>-GFP or dominant-negative inhibitors of NF $\kappa$ B in order to suppress the NF $\kappa$ B signaling in these clones and quantified the cytoplasmic tubulin aggregates by immunofluorescence-confocal microscopy (Figs. 12c and 13b). The results demonstrated that inhibition of NF $\kappa$ B signaling leads to suppression of cytoplasmic tubulin aggregates in these cells (Figs. 13a and b). The quantification of tubulin aggregates is shown in the micrograph in figure 13a and indicates that the suppression of NF $\kappa$ B transcriptional signaling causes suppression of aberrant MT dynamics and tubulin aggregates in ACH.p30<sup>II</sup> mutant GFP-positive cells expressing I $\kappa$ B $\alpha$ - S32A/S36A, I $\kappa$ K $\beta$  $\Delta$ 9, or I $\kappa$ K $\beta$  $\Delta$ 34 mutants. Acetylation of alpha-tubulin on lysine K40 is considered to be an indicator of microtubule stability (Howes SC et al, 2014). We sought to determine the acetylation levels of alpha-tubulin in HT1080 cells and the HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> mutant proviral clones, either in the absence or presence of nocodazole-treatment by immunoblotting and using an antibody against acetyl-tubulin which we compared to total tubulin levels in these cells (Fig. 13d). The results clearly demonstrate that alpha-tubulin acetylation was significantly lower in the ACH.p30<sup>II</sup> mutant clones, as compared to ACH.wt clones.



**Figure 14. Tax-G148V NF $\kappa$ B defective mutant exhibits reduced genomic instability and p65<sup>RelA</sup>-Stathmin molecular interactions.** **a.** HT1080 cells  $\pm$  nocodazole treatment were transfected with expression constructs for wt Tax or Tax-G148V mutant. Immunostaining was performed with Anti-Tubulin primary antibody and rhodamine red-conjugated secondary antibody and DAPI nuclear stain. Percent multinucleate cells per field were calculated by quantification of each visual field in triplicates using confocal immunofluorescence. **b.** HT1080 cells were co-transfected with pcDNA3.1-GFP and either wt Tax or Tax-G148V mutant and immunostained as described in 14a. Tubulin aggregates were quantified in GFP-positive cells (used as transfection control). **c. and d.** Representative images displaying tubulin aggregates are shown. Arrows in fig **c.** indicate tubulin aggregates. The quantification graphs in fig **d.** at right depict the relative fluorescence-intensities for the corresponding micrographs (drawn and analyzed using Zen 2.5D spatial analysis tool Carl Zeiss Microscopy). **e.** Immunoblots to determine the levels acetylated

tubulin as compared to total alpha-tubulin in HT1080 cells treated with nocodazole and transfected with expression constructs for wt Tax or Tax-G148V mutant. Untransfected HT1080 cells were used as control. **f.** Co-immunoprecipitation assay to study Stathmin- p65<sup>RelA</sup> complexes in 293 HEK cells transfected with wt Tax or Tax-G148V mutant. Input levels of proteins Stathmin, p65<sup>RelA</sup>, Tax, and Actin are shown in the upper panels.

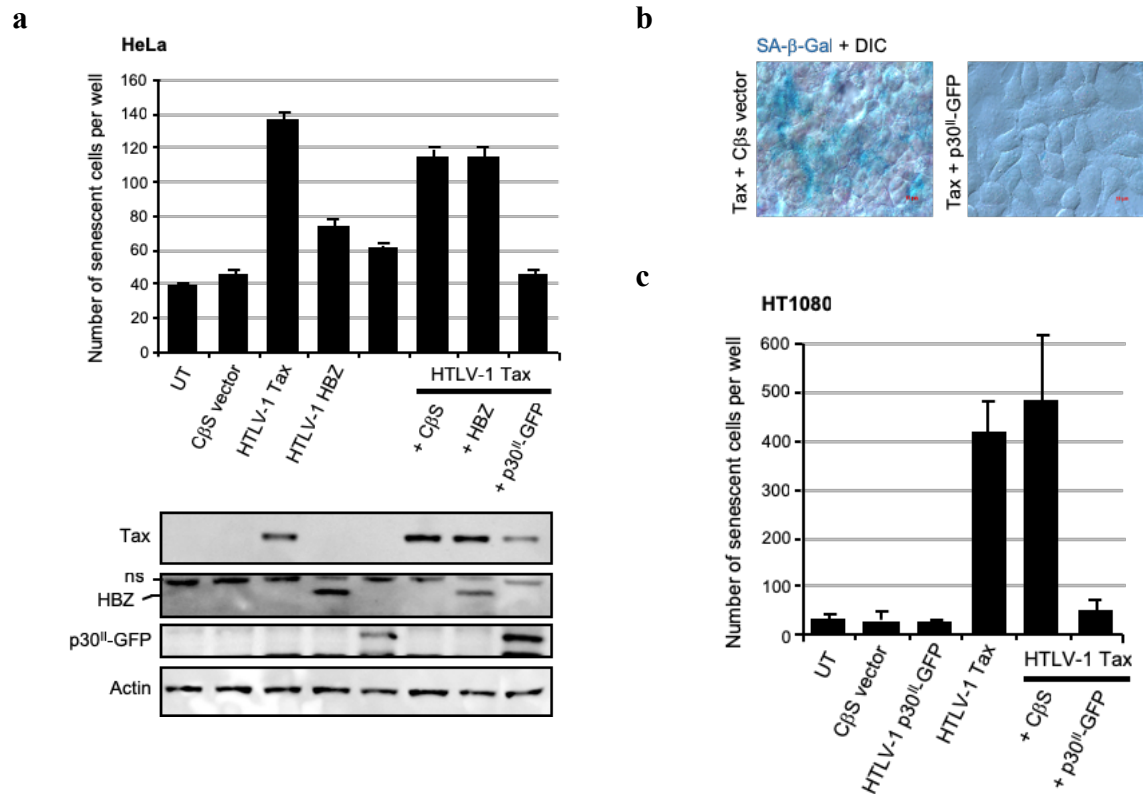
In order to assess whether the aforementioned genomic-instability and aberrant MT dynamics are a result of Tax-induced NFκB transactivation, I used the Tax-G148V mutant which is defective for NFκB transactivation (Yamaoka S et al, 1996), in the presence or absence of nocodazole treatment. HT1080 cells were co-transfected with pcDNA3.1-GFP and wt-Tax oncoprotein or Tax-G148V and stained using a primary antibody against alpha-tubulin and a fluorescent secondary antibody and DAPI nuclear stain. Using immunofluorescence-confocal imaging, I quantified the relative percent of GFP-positive (transfection control) cells with multinucleation or tubulin aggregates in untransfected HT1080 cells or HT1080 cells expressing either wt-Tax oncoprotein or the Tax-G148V (Figs. 14 a-d). The results in figures 14 a-d demonstrate that the wt-Tax induced significant tubulin aggregates/genomic-instability and multinucleation, even in the absence of nocodazole as compared to untransfected cells or the NFκB defective Tax-G148V. Upon studying the acetylated-tubulin levels by immunoblotting, we found that Tax-G148V mutant expressing cells had higher amounts of acetyl-tubulin compared to either wildtype Tax-expressing or untransfected cells (Fig. 14e), indicating much more stable MTs in NFκB deficient group. To detect the p65<sup>RelA</sup>-Stathmin/Op-18 complexes in the NFκB deficient Tax-G148V expressing cells, I performed co-immunoprecipitation assay in these cells with untransfected HT1080 cells and wt-Tax expressing cells as controls (Fig. 14f). The results demonstrated diminished p65<sup>RelA</sup>-Stathmin interactions in Tax-G148V as against wt-Tax (Fig. 14f). *These experiments establish a possible*

*link between p65<sup>RelA</sup>-Stathmin/Op-18 interactions as a result of Tax-induced NFκB transactivation and altered MT-dynamics and genomic-instability in Tax-expressing HTLV-1-infected cells.*

## **2.7 Tax-induced cytotoxicity can be countered by inhibition of NFκB-signaling**

Rationale: HTLV-1 Tax is the major oncogene responsible to drive HTLV-1 associated oncogenesis. Studies using Tax mutants defective for NFκB activation, defined that Tax-induced immortalization is highly dependent on its ability to constitutively activate NFκB dependent transcription (Smith MR and Green WC, 1990). Moreover, inhibition of NFκB was seen to prevent Tax-mediated cellular transformation and blocks the growth of HTLV-1 Tax-transformed cells and ATL cells, both in culture and in SCID mice (Yamaoka S et al, 1996; Satou Y et al, 2004). Together, these studies confirm that NFκB activation plays a crucial role in HTLV-1 Tax-mediated transformation in vitro. However, the constitutive NFκB activation by Tax also triggers a senescence and apoptosis response (Ho YK et al, 2012; Baydoun HH et al, 2015; Chaib-Mezrag H et al, 2012), suggesting that only those T-cells that are capable of overcoming NFκB-induced senescence and apoptosis can selectively undergo clonal expansion after HTLV-1 infection. Faulty apoptosis has been characterized as a major causative factor in cancer development and progression. HTLV-1 has been shown to suppress apoptosis in virus infected cells by viral protein interactions with host cellular factors (Saggioro D et al, 2009). In fact, HBZ has also been shown to suppress Tax-induced senescence by inhibiting NFκB signaling (Zhi et al, 2011). Together, these observations allude towards HBZ to modulate Tax-mediated viral replication and NFκB activation, thereby allowing HTLV-1-infected cells to proliferate and persist. HTLV-1 p30<sup>II</sup> and HBZ are both negative regulators of viral expression and transcription (Nicot C et al, 2004, Matsuoka M and Green PL, 2009), suggesting that there is a possibility that like HBZ, the HTLV-1 latency maintenance protein p30<sup>II</sup> also modulates Tax-induced NFκB activation and suppresses

Tax-induced cytotoxicity. *This led us to investigate whether p30<sup>II</sup> cooperates with the viral transactivator Tax and inhibits Tax-induced senescence and apoptosis and also investigate if it does so by repressing Stathmin and the NFκB-signaling pathway.*



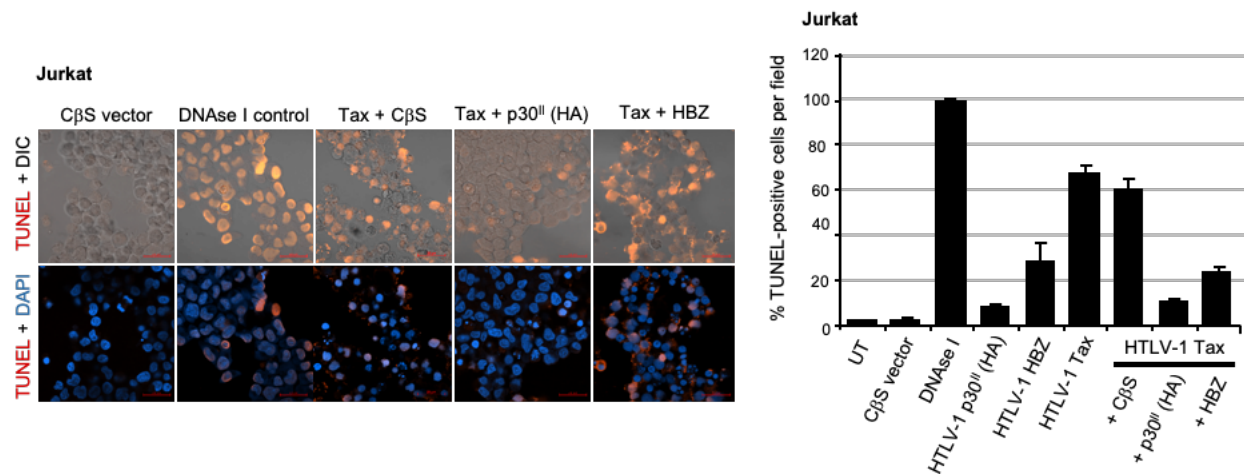
**Figure 15. HTLV-1 p30<sup>II</sup> counters Tax-induced cellular senescence.** **a.** HeLa cells were co-transfected with expression constructs for HTLV-1 p30<sup>II</sup>-GFP, wt Tax, CMV-HBZ and/or a CβS vector control, and then stained using X-Gal to detect senescence-associated Beta-galactosidase (SA-β-Gal; blue) after five days. The numbers of senescent SA-β-Gal-positive (blue) cells per well were quantified by counting each well in triplicate using color and DIC phase-contrast microscopy. Immunoblots were also performed to detect the expression of the HTLV-1 Tax, HBZ (Myc-tagged), p30<sup>II</sup>-GFP, and Actin **b.** Representative images (SA-β-Gal + DIC) are shown. **c.** Senescence staining was also performed on HT1080 fibrosarcoma cells that were co-transfected with HTLV-1 p30<sup>II</sup>-GFP, wt Tax, and/or a CβS empty vector control and the number of SA-β-Gal-positive cells per well were quantified in triplicates by microscopy.



Results and Discussion: Several studies showed that Tax-induced NF $\kappa$ B hyper-activation leads to over-expression of the cyclin-dependent kinase inhibitors, p21 and p27 which promotes an arrest of cell proliferation that triggers senescence. They propose that in HTLV-1 infected cells with impaired p21/p27 functions, the downregulation of NF $\kappa$ B by HBZ may suppress the senescence induced by Tax and hence promote clonal expansion of the infected cells (Kuo and Giam, 2006; Zhang et al, 2009; Zhi et al, 2011). We expounded upon this idea and investigated whether the latency protein p30<sup>II</sup> and/or HBZ could prevent Tax-induced cellular senescence. HeLa cells were cotransfected with various expression constructs for HTLV-1 Tax, p30<sup>II</sup>-GFP, HBZ (Myc-tagged), or an empty C $\beta$ S vector control. The senescent (blue) cells were visualized by using senescence-associated Beta-galactosidase (SA- $\beta$ -Gal) and staining the cultures with an X-Gal solution. The relative numbers of senescent (blue) cells per well were quantified by microscopy (Fig. 15a). It was observed that p30<sup>II</sup>-GFP suppressed Tax-induced cellular senescence more effectively as compared to the antisense HBZ protein under these experimental conditions (Fig. 15a). Immunoblotting was performed to detect the Tax, p30<sup>II</sup>-GFP, and HBZ (Myc-tagged) proteins (Fig. 15a, lower panels). Quantification of senescent cells was done by counting each sample in triplicates and representative micrographs for the same are provided in figure 15b. Additionally, we sought to replicate this experiment in HT1080 cells and demonstrated that p30<sup>II</sup>-GFP inhibited Tax-induced cellular senescence in cotransfected HT1080 cells (Fig. 15c).

Tax-activation of NF $\kappa$ B may also lead to cell death/apoptosis which is largely mediated by TNF-related apoptosis-inducing ligand (TRAIL) and requires an intact NF $\kappa$ B pathway activation (Rivera-Walsh I et al, 2001). However, it is unclear as to how the Tax-induced apoptosis is suppressed and converted to pro-tumorigenic for ATL development and progression. Hence, we sought to determine if the HTLV-1 latency maintenance protein p30<sup>II</sup> cooperates with the viral

transactivator by preventing Tax-induced apoptosis. For this experiment Jurkat E6.1 T-lymphocytes were cotransfected and fluorescent terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assays were performed. Certain samples were permeabilized and treated with ribonuclease-free deoxyribonuclease I (RNase-free DNase I) to consider as a positive control. The percent TUNEL positive cells were counted in triplicates using confocal-fluorescent imaging (Fig. 16) and representative microscopy images are shown in figure 14e. The results in figure 16 demonstrate that HTLV-1 Tax induces programmed cell-death and also HBZ induces apoptosis to a lesser extent. Indeed, as hypothesized, both p30<sup>II</sup> (HA-tagged) and HBZ effectively countered Tax-induced apoptosis in cotransfected cells (Fig. 16).

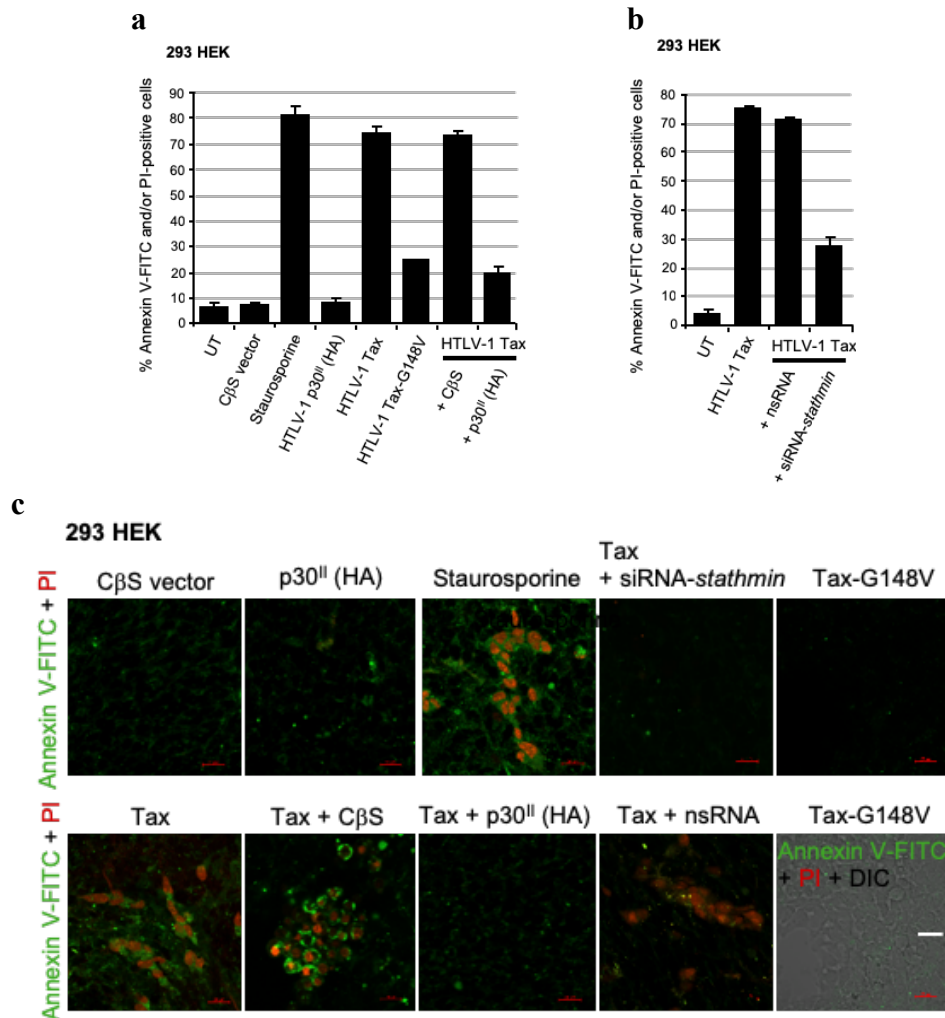


**Figure 16. HTLV-1 p30<sup>II</sup> inhibits apoptosis in Jurkat T-lymphocytes.** TUNEL staining was performed to study apoptosis in Jurkat T-lymphocytes co-transfected with expression constructs for wt Tax, HBZ, or p30<sup>II</sup>-GFP, or a CβS empty vector. Staining was performed using a Click-iT Alexa Fluor 594 TUNEL kit (Invitrogen). Representative microscopy images showing DAPI nuclear stain and TUNEL positive red staining are shown (left panels). Quantification of relative percentages of TUNEL-positive apoptotic cells per field was done by counting each field in triplicates using confocal microscopy (right panel).

Tax-G148V, the tax mutant defective for NFκB activation, is a very good system to determine if the apoptotic effect of wild-type Tax is dependent upon Tax-induced NFκB transcriptional activation. We used Annexin V-FITC and propidium iodide (PI) staining to compare the relative levels of apoptosis in 293 HEK cells expressing either the wt-Tax oncoprotein or the Tax-G148V mutant, and/or p30<sup>II</sup>-HA, or an empty CβS vector (Fig. 17a). As a positive control, some samples were treated with Staurosporine. The results were quantified as shown in figure 17a and representative microscopy images are shown in figure 17c. As is evident, the Tax oncoprotein induced more apoptosis (i.e., percentages of Annexin V-FITC and/or PI-positive cells) than the NFκB-defective Tax-G148V mutant or the p30<sup>II</sup> expression construct. Infact, the p30<sup>II</sup> protein countered the induction of cellular apoptosis by the viral transactivator Tax (Figs. 17 a and c). These results in figure 17, strongly suggest that apoptosis in HTLV-1 infected cells can be attributed to Tax-dependent NFκB signaling and that the viral latency protein p30<sup>II</sup> cooperates with Tax and counters Tax-induced cell death.

This revelation led to the next logical step to determine whether p30<sup>II</sup> counters Tax-induced apoptosis by repression of Stathmin. Hence, in order to determine whether inhibiting Stathmin could prevent Tax-induced cytotoxicity, 293 HEK cells were co-transfected with wt-Tax and a siRNA-stathmin oligonucleotide or nsRNA as a negative control, and then stained with Annexin V-FITC/PI and subsequently analyzed by confocal-microscopy. These results demonstrate that the inhibition of Stathmin levels countered Tax-induced apoptosis when compared to the nsRNA control (Figs. 17 b and c). *Taken together, these results indicate a strong possibility that Tax-induced NFκB hyperactivation leads to cytotoxic effects such as cell death and senescence and that HTLV-1 p30<sup>II</sup> cooperates with Tax to suppress the Tax-induced cytotoxicity through inhibiting Stathmin - a p53 dependent cofactor of NFκB/p65<sup>RelA</sup>. This cooperation between the viral*

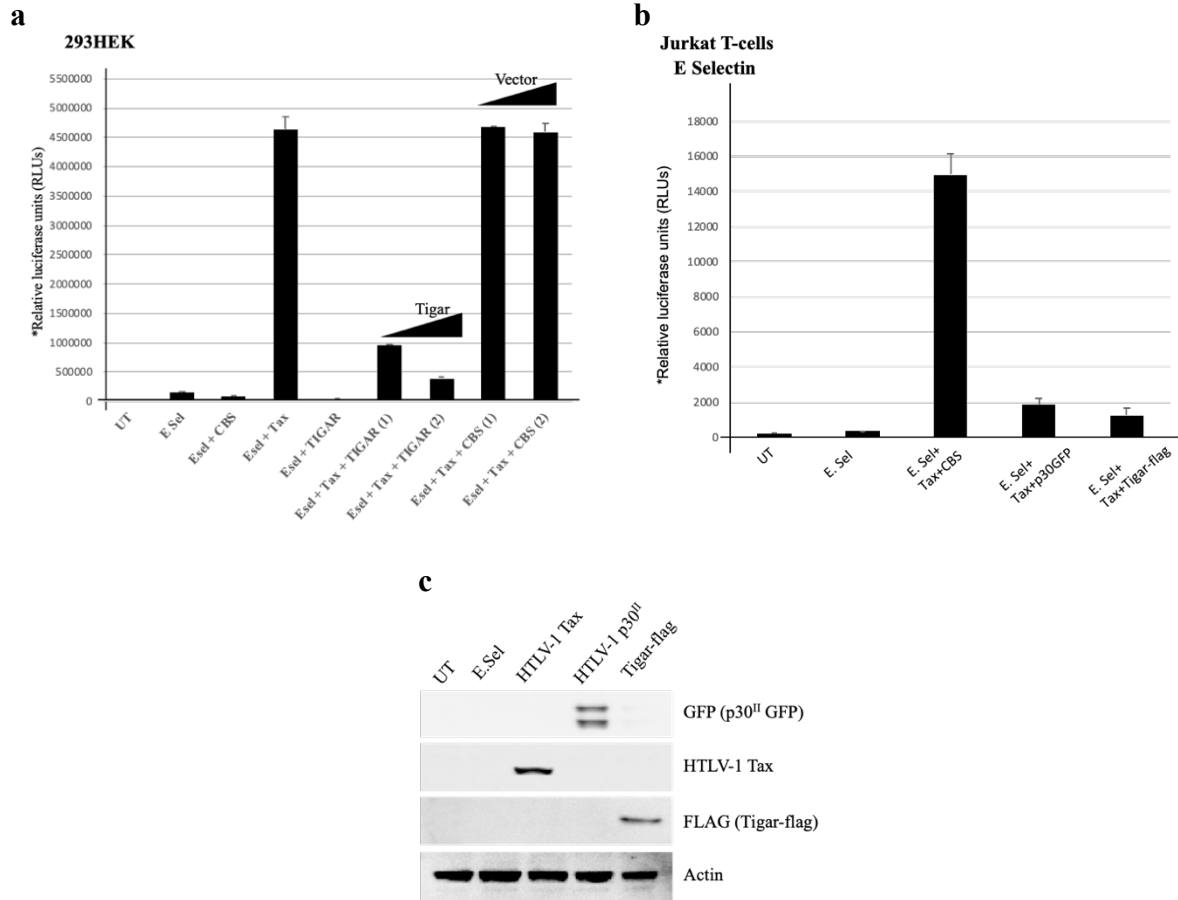
transactivator Tax and the latency protein p30<sup>II</sup> is imperative for ATL disease development in HTLV-1 infected cells.



**Figure 17. HTLV-1 p30<sup>II</sup> and Tax-G148V-NFκB defective mutant counter Tax-induced cytotoxicity by suppressing NFκB signaling and repression of Stathmin.** **a.** Quantification of percent apoptotic 293 HEK cells determined using Annexin V-FITC and propidium iodide (PI) staining. 293 HEK cells were co-transfected with wt Tax, Tax-G148V mutant, or p30<sup>II</sup> (HA-tagged) or a CβS empty vector and stained for apoptosis studies. Staurosporine treated cells were used as a positive control. Percentage of apoptotic cells were quantified by counting Annexin V-FITC and/or PI-positive cells in triplicates for each well by confocal microscopy. **b.** Apoptotic cells were also quantified in 293 HEK cells that were co-transfected with wt Tax and either a siRNA-stathmin or nsRNA control. **c.** Representative microscopy images are shown and DIC phase-contrast is provided in the merged image for the Tax-G148V mutant which exhibited reduced apoptosis.

## 2.8 HTLV-1 p30<sup>II</sup> induces TIGAR to suppress Tax-induced NFκB transactivation

Rationale: Ubiquitination is an important post-translational modification that plays a key role in regulation of NFκB activity (DiDonato J et al, 1996). Several studies have shown that Tax activates NFκB by activating the IκB kinase complex by generating Lys63-linked polyubiquitin chains. The IκK complex-associated Lys63/Met1-linked (K63/M1) hybrid polyubiquitin chains are generated through the Tax-mediated recruitment of linear ubiquitin chain assembly complex (LUBAC) to the IκK complex which results in *trans*-autophosphorylation-mediated IκK activation (Shibata Y et al, 2017). In fact, in one study, it was demonstrated that Tax recruits IκK complex to golgi associated compartments which is highly dependent on its ubiquitination and interaction with IκKγ (Harhaj NS et al, 2006). Another study recently showed that Tax induces generation of K63 and M1 chains by recruiting LUBAC to the IκK complex, leading to the formation of the active IκK complex (Shibata Y et al, 2017). Interestingly, Tang Y et al (2018) showed that TIGAR (Tp53-induced glycolysis and apoptosis regulator) suppresses NFκB-signaling by directly inhibiting LUBAC (linear ubiquitin chain assembly complex). They demonstrated that TIGAR competes with NEMO for association with the LUBAC and in doing so, prevented linear ubiquitination of NEMO which is essential for activation of IκKβ and other downstream targets resulting in potent inhibition of NFκB-dependent gene expression (Tang Y et al, 2018). Our lab recently demonstrated that p30<sup>II</sup> activates p53 and induces the expression of p53-regulated pro-survival signals, including TIGAR, which is required for its cooperation with cellular (e.g., c-Myc) and viral (Tax and HBZ) oncogenes (Hutchison et al, 2018; Romeo et al, 2018). ***We surmised that there is a possibility of p30<sup>II</sup> induction of TIGAR which leads to suppression of Tax-induced NFκB signaling in HTLV-1 infected cells.***



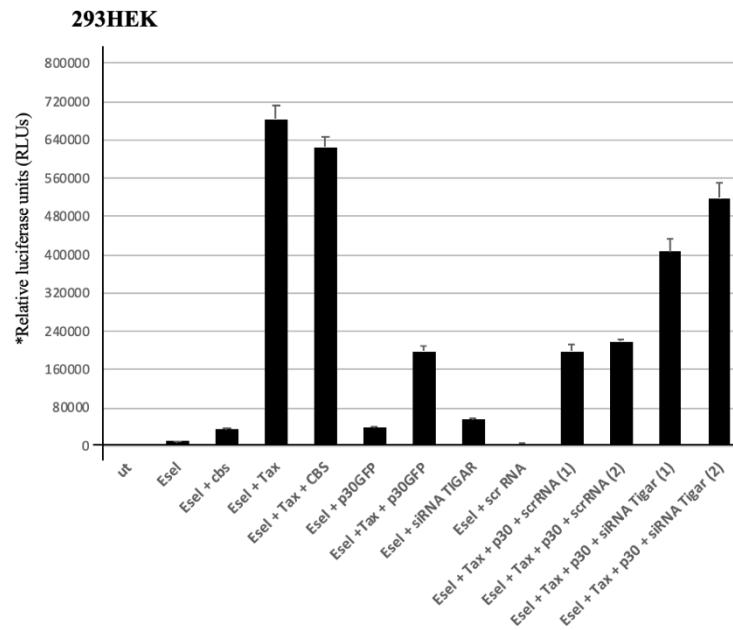
**Figure 18. TIGAR suppresses Tax-induced NFκB transactivation in 293 HEK cells and Jurkat T-lymphocytes.** **a.** 293 HEK cells were co-transfected with an *E. Selectin* luciferase construct and various expression constructs for wt Tax, TIGAR (flag-tagged), and a CβS vector control and relative luciferase activities were measured in triplicates and normalized to total cellular protein. **b.** Jurkat T-cells were co-transfected with an *E. Selectin* luciferase construct and various expression constructs for wt Tax, HTLV-1 p30<sup>II</sup>-GFP, TIGAR (flag-tagged), and a CβS vector control and relative luciferase activities were measured in triplicates and normalized to total cellular protein. **c.** Protein expression for wt Tax, HTLV-1 p30<sup>II</sup>-GFP, TIGAR (flag-tagged) was detected by immunoblotting and normalized to Actin.

Results and Discussion: TIGAR has been found to be overexpressed in several tumor cell types and cancers including colon cancer, breast cancer, nasopharyngeal carcinomas, adenomas, glioblastomas, and the hematological malignancy multiple myeloma (Lee et al, 2014) suggesting

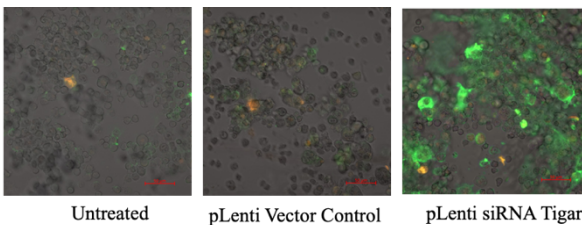
that TIGAR may function to promote cancer development. According to the Warburg effect, cancer cells require higher amounts of glucose and have an increased glycolysis owing to an increase in proliferation rate which requires large amounts of ATP, NADPH, and other metabolites, such as dNTPs (Jiang et al, 2013). However, increased cell growth and proliferation also leads to increased ROS meaning more oxidative stress and damage. This oxidative stress can be prevented by TIGAR which has been shown to reduce the amounts of fructose-2,6-bisphosphate leading to an accumulation of glucose-6-phosphate. The increase in glucose-6-phosphate shunts to the pentose phosphate pathway (PPP) and produces ribose-5-phosphate, a nucleotide precursor, and NADPH which is a ROS reducing agent (Bensaad et al, 2006; Bensaad et al, 2009; Jiang et al, 2013). TIGAR can promote cell survival and proliferation by decreasing oxidative stress and increasing the production of ribose for DNA and RNA synthesis. TIGAR was also reported to protect cells from genotoxic drug induced DNA damage partly through the regulation of pentose phosphate pathway products (NADPH and ribose) and reduction of reactive oxygen species (Yu HP et al, 2015). All these studies point towards cyto-protective role of TIGAR which can also protect HTLV-1 infected cells from Tax-induced cytotoxicity owing to constitutive NFκB-signaling by Tax and play an important role in HTLV-1 oncogenesis. In fact, Romeo et al (2018) showed that the induction of TIGAR by HTLV-1 p30<sup>II</sup> prevents ROS accumulation and senescence (Romeo M et al, 2018).

In order to assess if HTLV-1 p30<sup>II</sup> induction of TIGAR leads to suppression of NFκB-transcriptional activation by HTLV-1 Tax, we co-transfected 293 HEK cells and also Jurkat T-lymphocytes using an E. Selectin luciferase construct and various expression constructs for wt Tax, HTLV-1 p30<sup>II</sup>-GFP, TIGAR (flag-tagged), and a CβS vector control and measured relative luciferase activities were in triplicates. The luciferase reading was normalized to total cellular

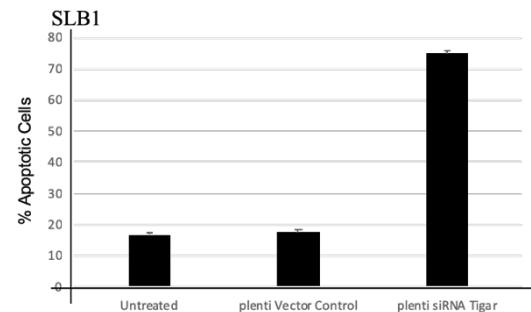
a



b



c



**Figure 19. TIGAR knockdown inhibits p30<sup>II</sup> suppression of Tax-induced NFκB activation and causes apoptosis in HTLV1 +ve SLB1 cells. a.** 293 HEK cells were co-transfected with E. Selectin luciferase reporter plasmid construct and various expression constructs for wt Tax, p30<sup>II</sup>-GFP and a siRNA-TIGAR or a non-specific scr-RNA in increasing concentrations. Relative luciferase values were measured in triplicates and normalized to total cellular protein. **b.** SLB1 T lymphocyte cells were transduced using lentiviral constructs for either an empty vector control or siRNA TIGAR (provided by T. Hutchison). Annexin/PI staining was performed to study apoptosis. Some samples were untreated SLB1 cells to use as control. **c.** Quantification of apoptosis from 19b. Annexin/PI stained samples were quantified by confocal microscopy and each field was counted in triplicates.

protein. Indeed, as expected we saw that TIGAR inhibited Tax-induced NFκB-transcriptional activation in a dose dependent manner (Fig. 18). Next, we knocked-down TIGAR protein



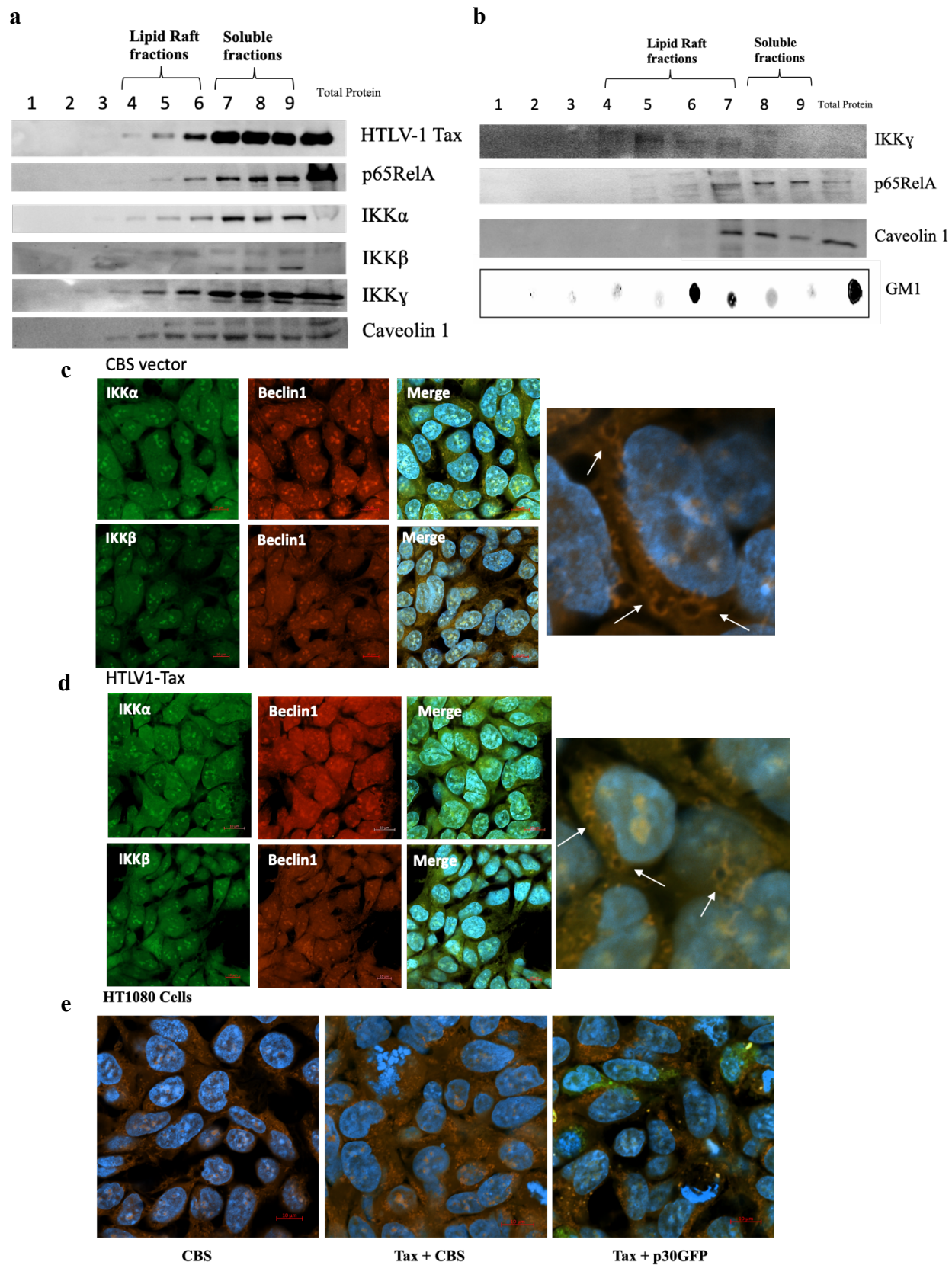
expression using siRNA against TIGAR and used a non-specific scrambled RNA control in 293 HEK cells. These cells were co-transfected using E. Selectin luciferase construct and various expression constructs for wt Tax, HTLV-1 p30<sup>II</sup>-GFP, TIGAR (flag-tagged), siRNA-TIGAR and a C $\beta$ S vector control and a ns scr-RNA control. The relative luciferase activities were measured in triplicates and normalized to total cellular protein (Fig. 19). We observed that knockdown of TIGAR expression in cell transfected with wt Tax and p30<sup>II</sup> GFP, rescued the p30<sup>II</sup> suppression of Tax-induced NF $\kappa$ B activity on E-selectin promoter (Fig 19). Finally, infectious SLB1 T cells were repeatedly transduced using lentiviral constructs for either an empty vector backbone or siRNA against TIGAR. These cells were then adhered on polyL/ConA treated slides and stained using Annexin/PI (BD Pharmingen) and subsequently observed by confocal imaging. Quantification of percent Annexin and/or PI positive cells revealed that TIGAR knockdown in HTLV1-positive SLB1 cells leads to much higher apoptosis and cell death when compared to untreated or vector control transduced cells. These results in figures 18 and 19 allude to the possibility that in an alternative mechanism, HTLV-1 p30<sup>II</sup> also suppresses Tax-induced cytotoxicity by induction of TIGAR. One possibility for the mechanism could be that TIGAR competes with Tax for the ubiquitination of IkK $\gamma$  and in doing so, prevents the Tax-IkK $\gamma$  interactions and suppresses NF $\kappa$ B hyperactivation and the cytotoxicity associated with the same. Further studies are however required to confirm these findings and deduce a possible mechanism.

## **2.9 HTLV-1 Tax targets the IkK complex to lipid rafts and p30<sup>II</sup> suppresses lipid rafts**

Rationale: NF $\kappa$ B is sequestered in the cytoplasm in an inactive form by the family of IkB proteins and in response to stimulation the NF $\kappa$ B/p65<sup>RelA</sup> gets rapidly activated and transports to the nucleus where it activates T-cell proliferation and a wide variety of genes controlling cell survival, development and differentiation. T-cell activation leads to the activation of NF $\kappa$ B in the

membrane microdomains, termed lipid rafts for promoting T-cell proliferation (Huang J et al, 2009). Lipid rafts are cholesterol- and sphingolipid-rich, detergent-resistant plasma membrane microdomains that are synthesized from the Golgi and recycled between the Golgi and the plasma membrane (Rocks O et al, 2005). Owing to the presence of both nuclear import and export signal peptides, the Tax protein is distributed between the cytoplasm as well as the nucleus (Alefantis T et al, 2003). This distribution pattern of Tax allows for its two major activities: activation of IκB kinases by the cytoplasmic Tax and in the nucleus it transactivates the HTLV-1 LTR through induction of the CREB activity. Tax exhibits differential modes of IκB kinase activation. However, it is still unclear whether Tax-mediated activation of NFκB is involved in lipid raft translocation of IκB kinases. It has been shown that cytoplasmic Tax mediates activation of IκB kinases and a portion of Tax was found to be in the Golgi where it recruits the IκK complex (Nejmeddine M et al, 2005). Huang J et al (2009) showed that the Tax protein accumulates in the Golgi-associated lipid rafts where it activates NFκB in both T cells and non-lymphoid cells by translocating IKK to the microdomains and primarily targeting IKKγ (Huang J et al, 2009). ***We sought to determine the presence of the IκK complex components in the lipid rafts in Tax expressing cells and also determine the effects of p30<sup>II</sup> upon Tax-induced lipid rafts.***

Results and Discussion: One of the several mechanisms through which HTLV-1 Tax can induce NFκB activation is through the recruitment of IKK signalosome to the lipid raft microdomains. Tax directs lipid raft translocation of IκK through selective interaction with IκKγ and accordingly, depletion of IκKγ impairs Tax-directed lipid raft recruitment of IκKα and IκKβ (Huang J et al, 2009). Ren T. (2015) suggest the possible role of Tax in utilizing lipid rafts as signaling platform where it recruits IκB kinases and autophagy molecules and activates NFκB and autophagy pathways (Ren T et al, 2015). They demonstrated that Tax activates the IκB kinase



**Figure 20. HTLV-1 Tax recruits the IKK complex to lipid rafts.** **a.** Immunoblots on SLB1 cells lipid raft fractions showing the expression of Tax, p65RelA, IKK $\alpha$ , IKK $\beta$ , IKK $\gamma$  and the lipid raft marker Caveolin 1. **b.** Immunoblots on Jurkat T-cells lipid raft fractions showing the expression of p65RelA, IKK $\gamma$  and the lipid raft marker Caveolin 1. A dot blot at the bottom of the panel shows the expression of GM1 lipid raft marker. **c and d.** Confocal immunofluorescence images of 293 HEK cells transfected either with empty vector or HTLV-1 Tax and immunostained for IKK $\alpha$  (Green) or IKK $\beta$  (Green) and Beclin1 (Red) (lipid raft marker) and DAPI nuclear stain. Inset shows enlarged images to show Merge images of IKK $\alpha$  and Beclin1 on lipid rafts (shown with white arrows). **e.** HT1080 cells were co-transfected with expression constructs for wt Tax and either a C $\beta$ S vector control or HTLV-1p30<sup>II</sup>-GFP and immunostained for Beclin1 (Red) and DAPI nuclear stain. p30<sup>II</sup>-GFP was visualized by direct GFP fluorescence (Green).

complex which leads to recruitment of an autophagy molecular complex containing Beclin1 and Bif-1 to lipid raft microdomains.

We used HTLV-1 +ve SLB1 cells for lipid raft fractionation and performed immunoblots to detect the presence of various IKK complex components (IKK $\alpha$ , IKK $\beta$ , IKK $\gamma$ ), HTLV-1 Tax, p65<sup>RelA</sup> and used Caveolin1 as a lipid raft marker. We observed that IKK $\alpha$ , IKK $\gamma$ , HTLV-1 Tax, p65<sup>RelA</sup> and to a lesser extent IKK $\beta$  were present in lipid raft fractions (fractions 4,5,6) (Fig. 20 a). We compared SLB1 fractions to Jurkat T-cells lipid raft fractions, and we observed that much lesser amount of IKK $\gamma$  and p65<sup>RelA</sup> was present in the lipid raft fractions (fractions 4-7) (Fig. 20 b). We also immunostained 293 HEK cells transfected with either HTLV-1 Tax or a C $\beta$ S vector, using Beclin1 (Rhodamine Red) and either IKK $\alpha$  or IKK $\beta$  (Green) to visualize lipid rafts in the presence or absence of Tax. We observed that there was a significant amount of colocalization of the IKK $\alpha$  or IKK $\beta$  with Beclin1 on lipid rafts in Tax-expressing cells and almost none in the other group (Figs. 20 c and 20 d; the white arrows indicate lipid raft microdomains in the image). The results in Fig. 20 a-d demonstrate that Tax indeed recruits the IKK complex components to lipid

raft microdomains. We next wanted to study the effects of HTLV-1 p30<sup>II</sup> upon Tax induced lipid rafts. We used HT1080 cells transfected with expression constructs for HTLV-1 Tax and a C $\beta$ S vector control or p30<sup>II</sup>-GFP and immunostained using Beclin1 (Red) and DAPI nuclear stain to visualize the lipid rafts (Fig. 20 e). We observed that while Tax induced the presence of several defined lipid raft microdomains in these cells, HTLV-1 p30<sup>II</sup> lead to disruption of or reduced lipid rafts in Tax-expressing cells (Fig. 20 e). *The results in figure 20 show promising preliminary data that Tax recruits IKK complex signalosome components to lipid raft microdomains and that there is a possibility that p30<sup>II</sup> disrupts Tax-lipid rafts which is probably another mechanism to suppress Tax-induced NF $\kappa$ B activation and reduce Tax-induced cytotoxicity in order to promote cell proliferation and survival. Further studies are required to deduce the exact mechanism of lipid raft activation of NF $\kappa$ B by Tax and its implications on HTLV-1 associated carcinogenesis.*

### **CHAPTER III**

#### **DISCUSSION AND OUTLOOK**

Viral infections are one of the major environmental factors that contribute to the etiology of cancer. Several human viruses are associated with cancer including human papillomavirus associated with cervical cancer, Hepatitis B and C viruses associated with hepatocellular cancer, Epstein Barr virus causing Burkitt's lymphoma and nasopharyngeal cancer, Kaposi's sarcoma herpesvirus causing Kaposi's sarcoma and human T-cell leukemia virus (HTLV-1) being the causative agent of adult T-cell leukemia/lymphoma (ATL). Of all these viruses, HTLV-1 is the only virus directly linked to a human cancer. HTLV-1 infects approximately 20 million individuals world-wide (Proietti et al, 2005). About 2–5% of these individuals develop ATL after a long latent period, alluding to a multistage process of immortalization and transformation of T-lymphocytes. Even though the mechanism of how HTLV-1 infection progresses from clinical latency to T-cell malignancy is not well understood but it is certain that it involves the unique viral transactivator, oncoprotein Tax. HTLV-1 Tax has been shown to be sufficient for immortalizing T-lymphocytes (Grassmann R et al, 1989) and transforming rat fibroblasts (Tanaka A et al, 1990). Furthermore, LTR-driven Tax transgenic mice expressing were found to develop neurofibromas - a tumor of mesenchymal tissue (Nirenberg M et al, 1987) and the T-cell specific, granzyme B promoter-driven Tax transgenic mice developed large granular lymphocytic leukemia (Grossman et al, 1995). Together, these studies confirm that HTLV-1 transactivator Tax plays a crucial role in cellular transformation and tumorigenesis. Tax is a 40kD nuclear phosphoprotein that has

pleiotropic activities and is a potent activator of HTLV-1 transcription and NF $\kappa$ B signaling pathways (Matsuoka M and Jeang KT, 2007). In response to immune stimuli, NF $\kappa$ B activation occurs transiently in normal T-cells and functions to carry out antigen-stimulated T-cell proliferation and survival. Contrarily, HTLV-1 Tax induces persistent activation of NF $\kappa$ B, causing deregulated expression of a large number of cellular genes which may ultimately lead to initiation of T-cell transformation. Tax activates NF $\kappa$ B pathway by signaling through the IKK complex (Sun SC and Yamaoka S, 2005). Tax binds to IKK $\gamma$  via lysine K63-linked polyubiquitin chains which causes phosphorylation and activation of IKK $\alpha$  and IKK $\beta$ . This leads to phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  and allows the NF $\kappa$ B/p65<sup>RelA</sup> subunit to be released and migrate into the nucleus (Sun SC and Ballard DW, 1999). Tax can also activate the alternative NF $\kappa$ B pathway by binding to an IKK $\alpha$ -IKK $\gamma$ -p100 complex and inducing the processing of NF $\kappa$ B/p100 to its active form p52 (Sun SC, 2011). Despite its crucial role in oncogenesis, the constitutive hyperactivation of NF $\kappa$ B signaling by Tax has been shown to be cytotoxic and triggers a senescence and apoptosis response (Ho YK et al, 2012; Baydoun HH et al, 2015; Chaib-Mezrag H et al, 2012). Previously it was shown that the HTLV-1 antisense protein HBZ can alleviate Tax-induced senescence by modulating the NF $\kappa$ B pathway (Zhi et al; 2011), we demonstrated in this study that the other pX-encoded viral proteins HBZ and p30<sup>II</sup> that inhibit NF $\kappa$ B transactivation cooperate with Tax and counter Tax-induced senescence and apoptosis (Hutchison T et al, 2018) (Figs. 7, 8, 9, 15, 16 and 17). A hallmark of cancer is variable clastogenic DNA damage and/or polyploid and aneuploid chromosomes (Loeb KR and Loeb LA, 2000). Clastogenic DNA changes and aneuploidy are frequently seen in ATL or Tax-expressing cells (Marriott SJ et al, 2002), and can be attributed to the abrogation of several cellular DNA-repair functions that monitor DNA structural damage in HTLV-1 infection. Aneuploidy is the result of either improper chromosomal

segregation during mitosis or from polyploidy created by failed cytokinesis. Aberrant centrosome duplication can also lead to aneuploidy by multipolar mitotic spindle formations (Peloponese Jr. JM et al, 2007). Tax has both clastogenic and aneuploidogenic effects in HTLV-1 infected cells. Tax inactivates cell cycle checkpoint controls and inhibits cellular DNA repair allowing DNA replication or chromosomal segregation to occur in the presence of damage and facilitates the accumulation of such genetic lesions that contribute to cellular transformation (Marriott SJ et al, 2002).

Stathmin is a microtubule destabilizing protein which is transcriptionally repressed by wild-type p53 by formation of p53/HDAC1/mSin3a transcriptional repressor complexes (Murphy M et al, 1999). One of the most frequent events in human cancers is the mutation or loss of p53 activity (Hollstein et al, 1994). Unlike most cancers HTLV-1 positive ATL clinical isolates mostly have a high level of wildtype p53 (Zane L et al, 2012), opening the possibility that in HTLV-1 pathogenesis the p53-target genes are subverted selectively to promote oncogenesis. The p53 tumor suppressor is the guardian of genome and functions in several cell signaling pathways including those for proliferation, DNA-damage repair, and apoptosis. Our lab has previously demonstrated the induction of p53 in p30<sup>II</sup>-expressing cells suggesting a role for p30<sup>II</sup> in manipulating survival versus apoptosis pathways controlled by p53 (Romeo M et al; 2018). We also recently demonstrated that the HTLV-1 latency protein p30<sup>II</sup> interacts with MYST-family acetyltransferase TIP60 and inhibits the lysine K120-acetylation of the p53 protein (Romeo M et al, 2018; Fig. 6b) which may lead to regulation and expression of p53 regulated pro-survival signals such as TIGAR (Romeo M et al, 2018; Hutchison T et al, 2018). Lu et al showed a novel oncogenic role of stathmin in aggressive pancreatic cancer by demonstrating that Stathmin binds to and stabilizes the NFκB/p65<sup>RelA</sup> subunit (Lu et al, 2014). In the present study, our experiments



demonstrate that the p30<sup>II</sup> protein dampens Tax-induced NFκB transcriptional activation by suppressing Stathmin expression and destabilizing NFκB/p65<sup>RelA</sup> subunit possibly through a p53-dependent pathway (Fig. 10). In consensus with Lu et al (2014), we saw robust interactions between the Stathmin protein and NFκB/p65<sup>RelA</sup> by co-immunoprecipitation assays in 293 HEK cells and HTLV-1 infected cell lines SLB1 and MJG11 (Fig. 9). To confirm the role of Stathmin as p65<sup>RelA</sup> cofactor, we used a siRNA-stathmin oligonucleotide to deplete Stathmin protein expression and indeed saw that Stathmin knockdown also destabilized p65<sup>RelA</sup> and inhibited Tax-dependent NFκB transactivation (Fig. 9). The siRNA-knockdown of Stathmin also inhibited the formation of p65<sup>RelA</sup>-Stathmin immune-complexes in the infectious HTLV-1 T-cell-line, SLB1 (Fig. 9). Since it is known that Stathmin is negatively regulated by p53 (Murphy M et al, 1999), we used p53-R175H; a dominant-negative DNA-binding mutant of p53 in 293 HEK cells (Fig. 10) and as expected we saw that the mutant p53 countered suppression of Stathmin by p30<sup>II</sup>-GFP. Interestingly though, contrary to our expectation, the p53-R175H mutant inhibited Tax-dependent NFκB transactivation from the E-selectin promoter (Fig. 10). One possible explanation could be that the mutant p53-R175H sterically hinders the binding of Tax to IκKγ subunit of the IκK complex through direct p53-Tax interactions (Yamaoka S et al, 1998; Pise-Masison CA et al, 1998, Pise-Masison CA et al, 2000a). It is also plausible that p53-R175H interferes with p65<sup>RelA</sup> transcriptional activation since Tax-induced NFκB signaling is required for p65<sup>RelA</sup> and p53 interactions (Jeong SJ et al, 2004; Pise-Masison CA et al, 2000b).

In the mid-1990s, a major breakthrough in HTLV-1 research came with the development of the ACH HTLV-1 molecular clones and its mutant clones, allowing for characterization of mutations of key viral proteins in the context of the entire virus (Bartoe JT et al, 2000; Silverman LR et al, 2004). We used the infectious HTLV-1 ACH.p30<sup>II</sup> mutant provirus, defective for p30<sup>II</sup>

production and saw that in the absence of p30<sup>II</sup> these cells exhibited increased NFκB-dependent transcription, aneuploidy, and multinucleation/genomic instability either in the absence or presence of nocodazole-treatment, compared to the wildtype ACH clones (Figs. 12 and 13). The higher levels of Stathmin expression and reduced levels of acetylated-Alpha-Tubulin detected by immunoblotting in the ACH.p30<sup>II</sup> mutant proviral clones along with increased cytoplasmic tubulin aggregates also correlated with increased MT-destabilization in the p30<sup>II</sup> deficient clones when compared to the wild-type clones. These results allude towards the possibility of increased genomic instability in the absence of p30<sup>II</sup> latency protein (Figs. 12 and 13). We also observed that the repression of Stathmin either by the HTLV-1 latency factor p30<sup>II</sup>, or its targeted knockdown using siRNA-stathmin was able to counter Tax-induced cellular senescence and apoptosis (Fig. 14; Ho YK et al, 2012; Baydoun HH et al, 2015; Chaib-Mezrag H et al, 2012). Additionally, the use of Tax-G148V mutant defective for NFκB transactivation provided evidence for the dependence of Tax-induced cytotoxicity (in the form of multinucleation, MT-destabilization and cytoplasmic tubulin aggregates, senescence and apoptosis) upon Tax-induced NFκB hyperactivation. We saw that the Tax-induced cytotoxicity was significantly reduced or countered in the presence of p30<sup>II</sup> protein or in the NFκB-defective Tax-G148V mutant (Fig. 14).

HTLV-1 Tax induces genetic instability, in the form of multinucleation, chromatin bridges, aneuploidy, telomere attrition, and clastogenic DNA damage (Baydoun HH et al, 2012). Tax plays a crucial role in oncogenesis, but its expression is often silenced in the majority of ATL due to genetic alterations or 5'-LTR DNA hypermethylation of the *tax* gene. Interestingly however, despite the loss of Tax, NFκB activation was seen to be persistently active in ATL. The reason behind this inconsistency can be contributed to somatic mutations in genes in the T/B-cell receptor (T/BCR) and NFκB signaling pathways (Harhaj EW and Giam CZ, 2018).

In addition to ATL leukemogenesis, studies of the HTLV viruses also provide an exceptional model for understanding basic pathogenic mechanisms of virus-host interactions and human oncogenesis. The persistent activation of the NF $\kappa$ B pathway maintained by certain viruses contributes to oncogenic transformation (Hiscott J et al, 2001). Several reasons make the NF $\kappa$ B pathway an attractive target to viral pathogens. NF $\kappa$ B activation is a rapid, immediate early (IE) event that occurs within minutes after exposure to a relevant signal. Moreover, it does not require de novo protein synthesis, and results in a strong transcriptional stimulation of several early viral as well as cellular genes. Additionally, NF $\kappa$ B has many important target genes that profoundly influence the host cell cycle, including growth factors, cytokines and cytokine receptors, and proto-oncogenes. NF $\kappa$ B contributes to DNA damage and induction of oncogenic mutations through inflammation-mediated production of reactive oxygen and nitrogen species (ROS and RNS). The Tax-induced constitutive activation of NF $\kappa$ B signaling results in increased expression of iNOS and NO which causes double-strand DNA-breaks in HTLV-1-infected cells (Baydoun HH et al, 2015). Moreover, NF $\kappa$ B activates c-Myc and PI3K to induce expression of human telomerase reverse transcriptase (hTERT) for the long-term proliferation and clonal expansion of HTLV-1-infected cells that have acquired chromosomal abnormalities (Marriott SJ and Semmes OJ, 2005; Sinha-Datta U et al, 2004). NF- $\kappa$ B also represses the tumor suppressor p53 at the protein level by phosphorylation and degradation or by inducing the expression of p53-inhibitor MDM2 (Jeong SJ et al, 2004). Tax also induces a physical interaction between RelA and p53, suggesting another mechanism for NF $\kappa$ B-mediated p53 inactivation (Jeong SJ et al, 2004). Interestingly, the NF $\kappa$ B pathway was shown to act as a barrier for tumor development by helping to maintain a senescent state and regulating DNA damage-repair in primary mouse embryo fibroblast (MEF) cultures (Wang J et al, 2009). Currently, it is largely unknown how the anti-tumor activity of NF $\kappa$ B is

suppressed and converted to be pro-tumorigenic for ATL development. One suggestion on this theory came from Wang et al (2009) who speculate that in pre-neoplastic cells undergoing stress-induced senescence, the NF $\kappa$ B functions as a tumor suppressor and maintains cells in a senescent state. However, once these cells acquire sufficient genetic changes, activated oncogenes might stimulate the tumor-promoting function of NF $\kappa$ B that mediates cell survival and proliferation.

Stathmin is frequently overexpressed in human cancers and Lu et al (2014) showed that Stathmin activates NF $\kappa$ B dependent pathways to exert its oncogenic effects in pancreatic cancer development (Lu et al, 2014). In this study, we demonstrated for the first time, the p65<sup>RelA</sup>/Stathmin molecular interactions by viral transactivator Tax-induced NF $\kappa$ B hyperactivation. These interactions may modulate the NF $\kappa$ B-pathway activity and its effector functions associated with cellular transformation and oncogenesis including cell-cycle control, DNA damage and chromosomal abnormalities, p53-dependent pathways, senescence and apoptosis. Additionally, NF $\kappa$ B pathway has been shown to play a role in promoting cell motility and epithelial-to-mesenchymal transition (EMT) (Maier HJ et al, 2010) which directly correlates to the MT-remodeling function of Stathmin. In light of the p65<sup>RelA</sup>/Stathmin interactions observed in HTLV-1 infected cells with Tax-induced NF $\kappa$ B activation, it is likely that the effects of these interactions on MT-dynamics and remodeling can manifest in the form of angiogenesis, tumor invasion, cell migration and metastasis promoting T-cell transformation and ATL disease progression.

These results demonstrate that the HTLV-1 latency maintenance factor p30<sup>II</sup> cooperates with the viral transactivator Tax by repressing Stathmin/OP18 - a p53 dependent cofactor of NF $\kappa$ B/p65<sup>RelA</sup> and suppresses Tax-induced NF $\kappa$ B signaling, apoptosis, senescence, multinucleation and genomic instability in HTLV-1-infected cells. Moreover, this study revealed



Presently, chemotherapies rely heavily on tumor suppressor p53 and p53-dependent apoptotic pathways to block or remove malignant cells. Unfortunately, ATL responds poorly to most prevalent chemotherapies. Also, p53 mutations are very rare in ATL patient isolates and those that present with mutated p53 have been seen to have a short survival time and poor prognosis (Hatta and Knoeffler, 2002). The majority of ATL patients present with acute or lymphomatous ATLL leading to a median survival of only 0.5–2.0 years, despite intensive chemotherapy treatment (Shimoyama M, 1991; Tsukasaki K et al, 2009). Host pathways that play an important part in cell transformation can be used as chemotherapeutic targets. A constitutively activated NFκB pathway represents such a target in the case of HTLV-1 mediated ATL. The NFκB pathway is a key regulator of cell cycle, apoptosis, cell proliferation and genomic instability and accumulating evidence supports the concept that NFκB targeted therapies sensitize ATL cells to apoptosis (Rauch D and Ratner L, 2011). Bortezomib is a non-specific inhibitor of the NFκB pathway that inhibits proliferation of Tax tumors cells ex vivo and sensitizes cells to apoptosis, however toxicity constraints limited the efficacy of the treatment. Other inhibitors of NFκB such as Oridonin, NIK-333, curcumin, fucoidan, histone-deacetylase inhibitors, have been reported to induce apoptosis in ATL cells by repressing the NFκB pathway (Rauch D and Ratner L, 2011). These findings show great promise to develop therapies for ATL that target NFκB. In fact, one plausible way to sensitize these highly chemoresistant cancer cells to therapy could be to target p65RelA-Stathmin/Op-18 molecular interactions in ATL cells.

## **CHAPTER IV**

### **EXPERIMENTAL PROCEDURES AND METHODS**

#### **4.1 Cell Culture**

293 Human embryonic kidney (HEK) cells (CRL-1573; ATCC, Manassas, VA) were cultured in in a humidified incubator at 37°C under 5% CO<sub>2</sub> using Eagle's Minimum Essential Medium (EMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Riverside, MO), 100 U/ml penicillin, 100 µg/ml streptomycin-sulfate, and 20 µg/ml gentamycin-sulfate (Life Technologies, Waltham, MA). HT-1080 fibrosarcoma cells (CCL-121; ATCC) were cultured in DMEM, 10% FBS, 100 U/ml penicillin/streptomycin sulfate on collagen treated flasks at 37°C in 5% CO<sub>2</sub>. HeLa cells (CCL-2; ATCC) were cultured at 37°C and 5% CO<sub>2</sub> in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated FBS and 100 U/ml penicillin. The Jurkat E6.1 T-lymphocytes (TIB-152; ATCC) were cultured in RPMI-1640 (ATCC), 20% FBS, 100 U/ml penicillin/streptomycin sulfate at 37°C in 10% CO<sub>2</sub>. Primary human peripheral blood mononuclear cells (hu-PBMCs) were isolated previously in the lab, after informed consent obtained from enrolled donors by the SMU Memorial Health Center from whole blood samples using a Ficoll gradient and were cultured in RPMI-1640, 20% FBS, 100u/ml penicillin/streptomycin sulfate, 50 U/ml hIL-2 at 37°C in 10% CO<sub>2</sub>. The HTLV-1-transformed MJG11 T-cell-line (CRL-8294; ATCC) was cultured in RPMI-1640 medium, supplemented with 20% FBS and antibiotics, at 37°C under 10% CO<sub>2</sub>. The HTLV-1-transformed SLB1 lymphoma T-cell-line (kindly provided by P. Green, The Ohio State University-Comprehensive Cancer Center)

was cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% FBS and antibiotics at 37°C and 10% CO<sub>2</sub> incubator. The infectious HTLV-1 ACH.wt or ACH.p30<sup>II</sup> mutant provirus expressing transiently-amplified HT1080 cell clones, were generated and kindly provided by T. Hutchison in the lab by transfecting 2x10<sup>5</sup> cells in 6-well tissue-culture plates with plasmids that contain the full-length ACH.wt or ACH.p30<sup>II</sup> proviral nucleotide sequences (Hutchison T et al; 2018, Romeo M et al; 2018, Pise-Masison CA et al; 1998) and then seeding the transfected cultured cells in 96-well microtiter plates followed by screening to quantify the amounts of extracellular HTLV-1 p19Gag core antigen released into the culture supernatants by performing Anti-HTLV-1 p19Gag enzyme-linked immunosorbent assays (ELISAs; Zeptometrix, Buffalo, NY). The supernatants were filtered using 0.22 µm cellulose acetate syringe filters (Sartorius, Goettingen, Germany). The extracellular p19Gag levels were measured relative to a HTLV-1 p19Gag protein standard using a Berthold Tristar microtiter plate-reader in Absorbance mode (Berthold Technologies, Oak Ridge, TN). The virus-producing HT1080/HTLV-1 ACH.wt and ACH.p30<sup>II</sup> clones were expanded and repeatedly passaged and the continuous production of infectious HTLV-1 particles was confirmed by performing Anti-HTLV-1 p19Gag ELISAs (Romeo M et al; 2018, Hutchison T et al; 2018). The HT1080/HTLV-1 ACH proviral clones were cultured in collagen treated flasks using high glucose (4.5 g/L) EMEM and supplemented with 10% FBS and antibiotics and incubated at 37°C under 5% CO<sub>2</sub>. All cell-lines were negative for mycoplasma contamination.

## **4.2 Plasmids and Antibodies**

Wild type Tax and Tax mutants were expressed using following plasmids that were kindly provided by C.Z. Giam (The Uniformed Services University of the Health Sciences): the R<sub>CMV</sub>-wildtype HTLV-1 Tax (Harrod R et al; 1998, Harrod R et al; 2000), R<sub>CMV</sub>-Tax-M22 and Tax-M47 mutants –impaired for NFκB- and CREB-dependent transactivation, respectively (Smith MR



and Greene WC; 1990), and the NF $\kappa$ B-defective Tax-G148V mutant (Yamaoka S et al; 1996). The pEGFP-N3-HTLV-1 p30<sup>II</sup>-GFP and pMH-HTLV-1 p30<sup>II</sup> (HA-tagged) expression constructs (Nicot C et al; 2004) were kindly provided by G. Franchini (NCI/NIH). J. Nyborg (Colorado State University) kindly provided the pGL2-Basic-HTLV-1 TRE-luciferase reporter plasmid (Giebler HA et al; 1997) and the pcDNA-HBZ-MycHis expression construct (Hiven P et al; 2005) was provided by I. Lemasson (East Carolina University). The NF $\kappa$ B-responsive E-Selectin promoter-luciferase reporter plasmid has been described by Hong S et al (2007). The  $\kappa$ B responsive luciferase construct pCV63-HIV-1 $\kappa$ B-LTR ( $\Delta$ TAR)-luciferase reporter construct contains nucleotides 345-531 of the HIV-1LAI promoter, spanning the two  $\kappa$ B-responsive elements and three SP1-binding sites with a deletion of the trinucleotide bulge of the TAR, which were inserted into pGL2-Basic (Promega, Madison, WI). pcDNA3.1-GFP was described by Nicot C and Harrod R (2000). The pcDNA3-FLAG-I $\kappa$ K $\beta$  $\Delta$ 9 mutant and I $\kappa$ K $\beta$  $\Delta$ 34 mutant expression constructs were described by Sylla BS et al (1998). The “super repressor” mutant of I $\kappa$ B $\alpha$  pRc-I $\kappa$ B $\alpha$ -S32A/S36A, defective for Ser32/Ser36-phosphorylation and degradation (DiDonato et al; 1996), was kindly provided by P. West (Texas A&M University). pCEP-wildtype p53 and the pCEP-p53-R175H mutant expression constructs (Hermeking H et al; 1996) and were provided by B. Vogelstein (Johns Hopkins University).

The following antibodies were used in this study for immunoblotting and immunofluorescence experiments: Santa Cruz Biotechnology (Dallas, TX) antibodies include, mouse monoclonal Anti-HTLV-1 Tax (1A3), rabbit polyclonal Anti-GFP (FL), mouse monoclonal Anti-NF-kappa-B p65RelA (F-6), rabbit polyclonal Anti-Stathmin/Op-18 (FL-149), rabbit polyclonal Anti-I $\kappa$ B $\alpha$  (C-15), mouse monoclonal Anti-p53 (DO-2), mouse monoclonal Anti-Alpha-Tubulin (TU-02), mouse monoclonal Anti-Acetylated-Alpha-Tubulin (6-11B-1), mouse monoclonal Anti-c-Myc (9E10),

and goat polyclonal Anti-Actin (C-11). Other antibodies used were: mouse monoclonal Anti-FLAG M2 (Sigma-Aldrich), fluorescein isothiocyanate (FITC)-conjugated Anti-Bromodeoxyuridine (FITC-Anti-BrdU; BD-Pharmingen, San Diego, CA). The fluorescent rhodamine red-conjugated Anti-Mouse IgG (H+L) secondary antibody and horseradish peroxidase (HRP)-conjugated secondary antibodies (Anti-Mouse IgG [H+L]-HRP; Anti-Rabbit IgG [H+L]-HRP; Anti-Goat IgG [H+L]-HRP) used for chemiluminescence-imaging and immunoblotting were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

#### **4.3 Immunoblotting and co-immunoprecipitations**

Immunoblotting/western-blotting was performed to detect protein expression. About 48-72 hrs post-transfection, the transfected cells were harvested and pelleted by centrifugation for 7 min at 260 x g at 4°C, washed 2X with PBS, pH 7.4, and the pellet was resuspended in 1X Reporter Lysis Buffer (Promega). Cell lysis was performed by repeated freeze-thawing over dry-ice and passaging through a 27-gauge tuberculin syringe needle. In order to resolve the protein lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the lysates were then centrifuged for 2 min at 5,000 x g at 4°C and mixed with 2X Laemmli Sample Buffer containing 2-mercaptoethanol (Biorad Laboratories, Hercules, CA) and heat-denatured at 95°C for 3 min, loaded on a 12.5% polyacrylamide gel with a 4% stacking layer. The proteins were transferred onto a 0.2 µm nitrocellulose membrane (Whatman, Maidstone, UK) using a model TE 77 PWR semi-dry blotting unit (Amersham Biosciences, Little Chalfont, UK). The membranes were blocked using blocking buffer (3% w/v bovine serum albumin and 0.5% v/v Tween-20 in PBS, pH 7.4) for 1 hour on a shaker and then incubated for 2 hrs in respective primary antibodies (diluted 1:1000 or 1:2000 in Blotto buffer: 50 mM Tris-Cl, pH 8.0, 2 mM CaCl<sub>2</sub>, 80 mM NaCl, 0.2% v/v IGEPAL-CA630, 0.02% w/v sodium azide, and 5% w/v nonfat dry milk) with gentle shaking.

Next, the membranes were washed 2X with blotto buffer for 10 min and incubated for 1 hr with the relevant HRP-conjugated secondary antibodies (diluted 1:500). Finally the membrane was washed 2X with blotto buffer and once with TMN solution (100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris-Cl, pH 9.5) for 10 min, and developed by chemiluminescence-based detection using Pierce ECL Western Blot Reagent (Thermo Scientific, Rockford, IL) on the ChemiDoc Touch imaging system (BioRad Laboratories).

In order to study protein interactions and protein complexes, co-immunoprecipitations were performed. For this the cells were lysed using 500 µl of RIPA buffer (0.15 M NaCl, 50 mM Tris-Cl, pH 7.4, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% SDS), containing 50 ng/ml each of the protease inhibitors: pepstatin, leupeptin, chymostatin, bestatin, and antipain-dihydrochloride (Roche Applied Science), by repeated freeze-thawing and either syringe-shearing or sonication over an ice-bath using a microtip probe and Misonix S-4000 model instrument set at 70% amplitude (for the HTLV-1-infected MJG11 and SLB1 ATLL cell-lines). Samples were centrifuged for 5 min at 5,000 x g at 4°C to remove the cell debris. For immunoprecipitation, 250 µl from each lysate was mixed with 20 µl of a 50% slurry of Protein-G-agarose and 3-5 µl of the primary antibodies overnight at 4°C with gentle rotation. These agarose-immune complexes were then pelleted by centrifugation for 5 min at 5,000 x g at 4°C followed by washing 2X with RIPA buffer and resuspended in 30µl of 2X Laemmli Sample Buffer with 2-mercaptoethanol. These samples were then heat denatured by incubating in a heat-block at 95°C for 5 mins and then the proteins were resolved and detected by SDS-PAGE and immunoblotting as described above.

#### **4.4 Transactivations and luciferase reporter gene assays**

Luciferase reporter plasmid constructs were used to study Tax-induced NFκB transcriptional activation and the effect of HTLV-1 p30<sup>II</sup> on the same. For these experiments 293 HEK cells were

co-transfected with either an E-Selectin promoter-luciferase reporter plasmid or HIV-1  $\kappa$ B-LTRLAI ( $\Delta$ TAR) promoter-luciferase reporter plasmid (0.25  $\mu$ g), and R<sub>c</sub>CMV-HTLV-1 Tax and/or an HTLV-1 p30<sup>II</sup>-GFP expression construct or empty C $\beta$ S vector control. The cells were harvested after 48 hours with 1X Reporter Lysis Buffer (Promega) and lysed with rapid freeze-thaw cycles and syringe shearing. The protein concentrations were determined using the Bradford microassay and spectrophotometric analysis at 595 nm. Luciferase assays were carried out with equivalent amounts of sample using luciferin (Promega) and read on a Berthold Lumat LB 9507 luminometer (Berthold Technologies), and the values were normalized to equivalent amounts of total cellular protein. To study the transactivation using Tax mutants and the mechanism through which HTLV-1 p30<sup>II</sup> repressed Tax-induced NF $\kappa$ B transcriptional activation, the cells were transfected with expression constructs for the Tax-M22, Tax-M47, or Tax-G148V transactivation mutants (Smith MR and Greene WC; 1990, Yamaoka S et al; 1996), or the dominant-negative p53 DNA-binding mutant, p53-R175H (Hermeking H et al; 1996), dominant-negative I $\kappa$ K $\beta$  mutants, I $\kappa$ K $\beta$  $\Delta$ 9 and I $\kappa$ K $\beta$  $\Delta$ 34 (Sylla BS et al; 1998), or a phosphorylation/degradation-defective I $\kappa$ B $\alpha$  “super-repressor” mutant, I $\kappa$ B $\alpha$ -S32A/S36A (DiDonato J et al; 1996). In an alternate way to induce NF $\kappa$ B transcriptional activation, 293 HEK cells were stimulated using 100 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), and these cells were cotransfected with an E-Selectin promoter-luciferase reporter plasmid and pEGFP-N3-HTLV-1 p30<sup>II</sup>-GFP. To study the effects of Stathmin knockdown on Tax-induced NF $\kappa$ B activation, 293 cells were repeatedly transfected with siRNA-stathmin to knockdown Stathmin/OP-18 expression or an nsRNA oligonucleotide as a negative control. These cells were also co-transfected with E. Selectin promoter and luciferase assays were carried out to measure NF $\kappa$ B transactivation. The CREB-dependent transactivation of the HTLV-1 promoter by Tax was demonstrated by co-transfecting

293 HEK cells with 0.25 µg of a pGL2-Basic-HTLV-1 TRE-luciferase reporter plasmid (Giebler HA et al; 1997). Dual luciferase assays were conducted to compare the relative levels of NFκB-dependent transactivation between the HT1080/HTLV-1 ACH.wt and HT1080/HTLV-1 ACH.p30<sup>II</sup> mutant proviral clones. These cells were cotransfected with E-Selectin promoter-firefly-luciferase (or HIV-1 κB-LTR (ΔTAR)-firefly-luciferase) and tk-renilla-luciferase reporter plasmids and the dual-luciferase assays were performed using a Dual-Glo kit as recommended by the manufacturer's protocol (Promega). The RLU values from κB-dependent E-Selectin promoter-driven firefly-luciferase were normalized to equivalent tk-renilla-luciferase activities.

#### **4.5 siRNA knockdown of Stathmin**

Stathmin expression was inhibited using either a 2'-O-methyl-uridine-modified siRNA-stathmin (#1) oligonucleotide with 5' and 3' terminal phosphorothioate linkages and the sequence: 5'-mUCCAGUUCUUUCACCUGGAUAUC-3', or a bridged nucleic acid (BNA) siRNA-stathmin (#2) oligonucleotide with phosphorothioate linkages at the 5' and 3' ends with the sequence: 5'-AAUCAGCUCAAAGCCUGGCCU-3' (Biosynthesis, Lewisville, TX), using the HiPerFect transfection reagent (Qiagen, Germantown, MD) as per the manufacturer's recommended protocol. As a negative control, a 2'-O-methyl-uridine-modified non-specific RNA (nsRNA) oligonucleotide with the sequence: 5'-UUACCGAGACCGUACGUAU-3' (Biosynthesis) was used. Stathmin expression was inhibited in HTLV-1-transformed SLB1 T-lymphoblasts or 293 HEK cells by repeated transfections using using Qiagen Hi-perfect transfection reagent. We used SDS-PAGE and immunoblotting using a rabbit polyclonal Anti-Stathmin/Op-18 primary antibody (Santa Cruz Biotechnology) to confirm the depletion of Stathmin protein expression.

#### **4.6 Multinucleation and tubulin aggregates studies using confocal microscopy and flow cytometry**

Tax interacts with cell cycle components and often results in dysregulation causing cellular abnormalities, aneuploidy and immortalization of T-cells which ultimately lead to ATL (Curren R et al, 2012). In order to study the effects of Tax-induced NF $\kappa$ B signaling upon genomic instability and the role of p30<sup>II</sup> expression in HTLV-1-infected cells I quantified multinucleation and tubulin aggregates in HT1080 human fibrosarcoma cells, or the HT1080/HTLV-1 ACH.wt and HT1080/HTLV-1 ACH.p30<sup>II</sup> mutant proviral clones (Hutchison T et al; 2018; Romeo M et al; 2018) which were treated with the microtubule-depolymerizing agent, nocodazole (400 ng/ml; Sigma-Aldrich), for 24 hrs to induce metaphase (M-phase)-arrest. These cells were plated on collagen treated sterile glass coverslips in 35 mm<sup>2</sup> tissue-culture dishes, treated with 400ng/ml of nocodazole and incubated at 37°C and 5% CO<sub>2</sub>. After 24 hrs, the cells were fixed in 0.2% glutaraldehyde-1% formaldehyde in PBS for 15 min, washed 2X with PBS, and then incubated in Blocking buffer with gentle shaking. Immunostaining was performed, using a monoclonal Anti-Tubulin primary antibody (diluted 1:2000 in Blotto buffer) and a rhodamine red-conjugated Anti-Mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories; diluted 1:200) and DAPI nuclear stain (4',6-diamidine-2'-phenylindole dihydrochloride, DAPI; Invitrogen, Carlsbad, CA). Confocal immunofluorescence-microscopy was performed to determine the relative percentages of multinucleate cells, that had bypassed nocodazole-induced M-phase arrest, per field by counting visual fields in triplicates. We used the Tax-G148V NF $\kappa$ B defective mutant to determine whether it induces genomic instability compared to the wildtype Tax protein. For this, 1x10<sup>5</sup> HT1080 cells were plated on glass coverslips and transfected with R<sub>CMV</sub> expression constructs for wildtype HTLV-1 Tax or the Tax-G148V mutant. We used nocodazole to induce M-phase arrest. These slides were then fixed, blocked and immunostained, and confocal

microscopy was performed as described above to quantify the relative percentages of multinucleate cells per field.

I used flow cytometry to deduce the effects of HTLV-1 p30<sup>II</sup> on Tax-induced genomic instability. The HT1080 fibrosarcoma cells and HT1080/HTLV-1 ACH.wt or ACH.p30<sup>II</sup> mutant proviruses, were seeded at a density of  $2 \times 10^5$  cells/well and co-transfected with a pLenti-6.2/V5-DEST-HTLV-1 p30<sup>II</sup> (HA-tagged) expression construct or empty C $\beta$ S vector control in 6-well tissue culture plates for 72 hrs. Next, these cultures were labeled with 10  $\mu$ M BrdU for 6 hrs in EMEM supplemented with 20% FBS and immunostained using an Anti-BrdU antibody conjugated with fluorescein isothiocyanate (FITC; BD-Pharmingen). Following BrdU labelling, the cells were permeabilized and stained using the FITC-Anti-BrdU antibody; and the total genomic DNA content was quantified by staining the cultures with 7-aminoactinomycin D (7-AAD; BD-Pharmingen). Flow cytometry was performed on a Becton Dickinson FACS Caliber instrument (Becton Dickinson, Franklin Lakes, NJ). The samples were gated during acquisition to exclude sub-G1 cellular debris and apoptotic fragments. The flow cytometry data were analyzed using ModFit LT 3.0 software (Verity Software House, Topsham, ME).

The effects of NF $\kappa$ B-signaling induced by HTLV-1 Tax and the repression of Stathmin expression by p30<sup>II</sup> upon tubulin dynamics and the MT network were determined by confocal immunofluorescence microscopy in immunostained HT1080/HTLV-1 ACH.wt and HT1080/HTLV-1 ACH.p30<sup>II</sup> mutant proviral clones. These cells were plated on sterile glass coverslips in collagen treated 35 mm<sup>2</sup> tissue-culture dishes and then co-transfected using Qiagen Superfect with pcDNA3.1-GFP or pEGFP-N3-HTLV-1 p30<sup>II</sup>-GFP and/or various expression constructs for the dominant-negative I $\kappa$ K $\beta$  deletion mutants: I $\kappa$ K $\beta$  $\Delta$ 9 and I $\kappa$ K $\beta$  $\Delta$ 34, or the I $\kappa$ B $\alpha$  “super repressor” mutant, I $\kappa$ B $\alpha$ - S32A/S36A. After 72 hrs in culture in EMEM substituted with

10% FBS at 37 C and 5% CO<sub>2</sub>, the samples were fixed with 0.2% glutaraldehyde-1% formaldehyde in PBS for 15 min at room temperature, washed 2X with PBS, and incubated on gentle shaker in blocking buffer for 1 hr. The samples were then immunostained with a monoclonal Anti-Tubulin primary antibody and a rhodamine-red-conjugated Anti-Mouse IgG (H+L) secondary antibody and DAPI nuclear-stain. Confocal immunofluorescence microscopy was performed and the relative percentages of GFP-positive cells with cytoplasmic tubulin aggregates were quantified by counting each slide in-triplicate. I also used the HT1080 cells co-transfected with pcDNA3.1- GFP and wildtype HTLV-1 Tax or the Tax-G148V mutant which is NF- $\kappa$ B transactivation defective mutant and followed the above protocol to quantify GFP-positive cells with tubulin aggregates, to determine the effects of Tax-dependent NF- $\kappa$ B signaling on tubulin polymer dynamics. The relative fluorescence-intensities of the DAPI, GFP, and Tubulin-specific (red) signals were measured using the Zen 2.5D analysis tool (Carl Zeiss Microscopy, Jena, Germany).

#### **4.7 Senescence and Apoptosis**

Detection of senescence associated  $\beta$ -galactosidase was done to determine the effects of HTLV-1 p30<sup>II</sup> and HBZ upon Tax-induced cellular senescence (Ho YK et al; 2012). HeLa cells were plated on 60 mm<sup>2</sup> dishes (Grenier) and transfected with pRcCMV-HTLV-1 Tax, pEGFP-N3-HTLV-1 p30II-GFP, pcDNA-HBZ-MycHis, or an empty C $\beta$ S vector control. The transfected cells were cultured for four days in DMEM substituted with 10% FBS and antibiotics at 37°C and 5% CO<sub>2</sub> followed by serum starvation (0.05% FBS) for 24 hrs. The cells were stained using an adapted Cell Signaling Senescence  $\beta$ -galactosidase Cell Staining protocol using X-Gal (Sigma-Aldrich) to detect senescence-associated Beta-galactosidase (SA- $\beta$ -Gal) expression. The cells were fixed for 15 mins using fixative (PBS, 2% formaldehyde, 0.2% glutaraldehyde) and then washed with PBS



and stained using the  $\beta$ -galactosidase staining solution (930  $\mu$ L 1X staining solution [40mM citric acid/sodium phosphate (pH 6.0, 0.15 M NaCl, 2 mM MgCl<sub>2</sub>], 10  $\mu$ L Staining Supplement A [500 mM potassium ferrocyanide], 10  $\mu$ L Staining Supplement B [500 mM potassium ferricyanide], 50  $\mu$ L 20 mg/mL X-gal in DMF). These stained plates were incubated at 37°C overnight in a dry incubator without CO<sub>2</sub>. The numbers of blue senescent cells were quantified by counting visual fields in triplicates using a DIC filter on a Zeiss AxioImager Z2 fluorescence-microscope equipped with AxioCam MRc color and HRm monochromatic cameras and a Plan-Apochromat 20x/0.8 objective lens. SDS-PAGE and immunoblotting were performed to confirm the expression of Tax, HBZ (Myc-tagged), and p30<sup>II</sup>GFP. Direct fluorescence was used to visualize the p30<sup>II</sup>-GFP fusion and also to assess transfection control.

For apoptosis studies I used either Annexin staining or TUNEL staining in 293 HEK and Jurkat T-cells. 293 HEK cells were plated on 8-well glass chamber slides and co-transfected with 0.25  $\mu$ g of RcCMV-wildtype HTLV-1 Tax or the NF $\kappa$ B-defective Tax-G148V mutant, pMH-HTLV-1 p30<sup>II</sup> (HA-tagged), or a C $\beta$ S empty vector in various combinations using Qiagen Superfect reagent. To study the effects of inhibiting Stathmin expression on Tax-induced apoptosis, some cells were co-transfected with 50 ng of a siRNA-stathmin (#2) oligonucleotide or nsRNA as a negative control using Qiagen Hiperfect reagent along with wildtype Tax expression plasmid construct. Cells were stained after 72 hrs in culture using Annexin V-FITC staining kit (BD-Pharmingen - Annexin V conjugated to FITC) and propidium iodide (PI; BD-Pharmingen), and then using confocal fluorescence-microscopy the relative percentages of apoptotic (i.e., Annexin V-FITC and/or PI-positive) cells per well were quantified by counting in-triplicate. As a positive control, staurosporine-treated cells (12 nM for 4 hrs; Sigma-Aldrich) were included.

Apoptosis studies were also conducted using the fluorescence-based, terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) experiments. Jurkat T-cells were grown in suspension in a T-75 flask in complete medium (RPMI with 20% FBS and antibiotics). Cells were transfected by electroporation with 2.5 µg each of expression plasmids including CβS vector, RcCMV-HTLV-1 Tax, pEGFP-N3-HTLV-1 p30<sup>H</sup>-GFP, and pcDNA-HBZ-MycHis expression constructs or a CβS empty vector control using Bio-Rad Gene Pulser MXcell shock pod. The cells were pelleted by centrifuging for 7 mins at 2100 rpm at 4°C and washed with PBS. 2.5 x 10<sup>6</sup> cells were resuspended in 500 µl of ice-cold Bio-Rad Gene Pulser electroporation buffer in 0.4 cm gap Bio-Rad Gene Pulser cuvettes along with respective plasmid DNA and pulsed 2X for 10 microseconds duration at 250 Volts, 1,500 µF capacitance and 1000 Ohms resistance. The cells from each cuvette were then transferred to six-well plates and fed with 2ml of RPMI media substituted with 20% FBS and antibiotics and incubated for 48 hrs at 37°C and 10% CO<sub>2</sub>. After 48 hrs, the cells were adhered on slides treated with a 0.1 mg/ml Concanavalin A (Sigma)/poly-L-lysine by incubating for 30 minutes at 37°C. The Click-iT® Plus TUNEL assay kit Alexa Fluor 594 Imaging Assay from Invitrogen was used to detect apoptotic cells. The cells were washed with PBS and fixed (4% paraformaldehyde solution). 0.25% Triton X solution in PBS was used to permeabilize the fixed cells by treating for 20 minutes at 37°C. Cells were washed 2X with DI water. DNase I (Qiagen) solution (0.27U/µl) was used to treat cells for positive control. To perform the Click-iT reaction, TUNEL reaction cocktail was used to incubate cells for 30 mins at 37°C in dark. Cells were washed again with blocking buffer and treated with DAPI for 15 mins and observed. The relative percentages of fluorescent TUNEL-positive apoptotic cells per field were quantified by counting triplicate visual fields using confocal microscopy.

#### 4.8 Confocal microscopy

Confocal fluorescence-microscopy was used to visualize tubulin aggregates, multinucleation, Annexin V-FITC/PI or Alexa Fluor 594 TUNEL-staining in HTLV-1 Tax-expressing cells. These studies were performed on a Zeiss LSM800 instrument with an Airyscan super-resolution detector and stage CO<sub>2</sub> incubator (Carl Zeiss Microscopy). All images were taken using either Plan-Apochromat 40x/1.3 or Plan-Apochromat 63x/1.4 oil immersion objectives and Zeiss ZEN system software. The expression of HTLV-1 p30<sup>II</sup>-GFP in co-transfected cells was visualized on an inverted Nikon Eclipse TE2000-U microscope and D-Eclipse C1 confocal system (Nikon Instruments, Melville, NY) equipped with 633 nm and 543 nm He/Ne and 488 nm Ar lasers using a Plan Apo 20x/0.75 objective lens.

#### 4.9 Lipid raft isolation

Lipid rafts are cholesterol and sphingolipid-rich structures or microdomains of the plasma membrane, endoplasmic reticulum and golgi complex. I performed lipid raft isolation to study the effect of Tax-induced NFκB activation on localization of the IKK complex components in lipid raft microdomains (Ren T et al; 2015). Their isolation method is based on insolubility of lipids in ice-cold Triton X-100 followed by their separation on an OptiPrep density gradient. I used the Caveolae/Rafts isolation kit from Sigma-Aldrich. Jurkat T-cells or infectious HTLV-1 positive SLB1 cells were cultured in RPMI or IMDM with 10% FBS and antibiotic and ~ 3-5X10<sup>7</sup> cells were seeded in T-125 flasks. Cells were collected on ice in precooled 50 ml tubes and centrifuged at 450 x g at 4°C for 5 mins. The cell pellet was gently washed 2X with 10 ml ice cold PBS. Next, the cell pellet was gently resuspended in 2ml of diluted CTB-HRP (cholera toxin B subunit from *Vibrio cholerae*-peroxidase conjugate, Sigma C3741) solution and gently shaken on ice for 1 hr. The cells are then centrifuged, and the cell pellet is washed 2X with ice cold PBS. Cell pellet is

resuspended in 1ml lysis buffer containing 1% Triton X-100 and the cell lysate was transferred to a pre-cooled microcentrifuge tube and incubated on ice in cold room for 30 mins. 50 µl of lysate was kept aside as a positive control. Density gradient was prepared using different volumes of cell lysate, lysis buffer and OptiPrep solution with final OptiPrep concentrations as follows: (from bottom) 35%, 30%, 25%, 20%, 0% (top) and layered in precooled ultracentrifuge tubes. Balanced ultracentrifuge tubes were centrifuged at ~200,000 x g ultracentrifuged in a Beckman 70.1 Ti rotor for 4 hours at 4°C and then each 1 ml fraction from top to bottom was collected in separate microcentrifuge tubes. CTB-HRP was detected using dot blot analysis. Nitrocellulose membrane was placed on a PBS soaked Whatman filter paper and 2-3 µl sample from each fraction was placed along with original positive control lysate on the membrane. The membrane was allowed to air dry and washed once briefly with PBS and developed by chemiluminescence-based detection using Pierce ECL Western Blot Reagent (Thermo Scientific, Rockford, IL) on the ChemiDoc Touch imaging system (BioRad Laboratories). Anti-Caveolin-1 primary antibody and anti-rabbit secondary antibody was used for immunoblotting as a marker for lipid raft caveolae fractions. HTLV-1 Tax, p65<sup>RelA</sup>, IKKα, IKKβ, IKKγ were also detected using immunoblotting in lipid raft fractions.

## APPENDIX A

### **Generation of lentiviral constructs**

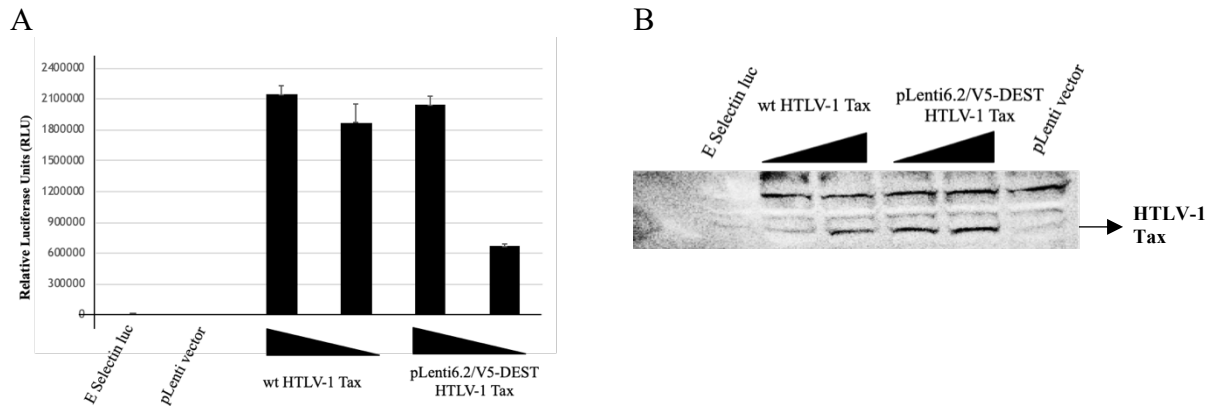
We use several different kinds of cell lines in our research studies and one of the most common methods used in biological research is protein expression which is achieved by transfecting plasmid DNAs in cells. Each cell line varies in its transfection efficiency, cell lines like HeLa (ATCC CCL-2) and 293 HEK (ATCC CRL-1573) are known for their ease of transfection. Contrary to this, cells that are not transformed by viruses or through cancer are generally more difficult to transfect. These hard to transfect cells can be transduced using a lentivirus which will infect individual cells and deliver the gene of interest with transduction efficiencies close to 100%. By constructing lentiviral vectors, I will be able to transduce and express my proteins of interest in primary hu-PBMCs which otherwise have extremely low transfection efficiency.

*Method for lentiviral production:* The lentiviral expression constructs were generated using the Invitrogen (ThermoFischer Scientific) ViraPower Gateway Expression Kit. This Invitrogen kit allows us to clone our gene of interest into a Gateway® entry vector to create an entry clone. An expression clone is generated by LR recombination reaction between the entry clone and a destination vector (pLenti6.2/V5-DEST). And finally, this expression clone is used ViraPower lentiviral expression system to produce virus particles containing our gene of interest.

The HTLV-1 Tax insert was PCR-amplified, gel purified, and ligated into the Invitrogen pCR8/GW/TOPO vector. The Tax insert was amplified from the plasmid RcCMV-wildtype HTLV-1 Tax (provided by C.Z. Giam) using the Tax-F (5' GGT ACC ACC ATG GCC CAC TTC

CCA GGG TTT GGA 3') and Tax-R (5' GC GGC CGC TCA GAC TTC TGT TTC TCG GAA ATG 3') forward and reverse oligonucleotide primers. The ligation reaction was performed using TOP10 E.coli strain (Invitrogen) and the Tax insert was ligated into the pCR8/GW/TOPO vector. Screening of the clones was performed by EcoR1 (NEB) restriction enzyme digestion and PCR-amplification was done to determine if the insert is present and also to ensure that the insert is in the correct orientation within the pCR8 backbone. The Tax clones #B5 and #B9 were found to have correct insert. To verify the orientation of the Tax insert, the Tax-F and GW2 (5' GTT GCA ACA AAT TGA TGA GCA ATT A 3'; Invitrogen) oligonucleotide primers were used. Clones B5 and B9 were then sequenced using the GW1 (5'-GTT GCA ACA AAT TGA TGA GCA ATG C-3'; Invitrogen) and GW2 primers. Next, the selected clones were inserted into the Invitrogen pLenti6.2/v5- DEST vector by homologous recombination using the provided Clonase II enzyme mix to generate a lentiviral construct that contains the gene of interest. The lentiviral plasmid construct which contains an insert will be transformed into StbI3 E. coli (designed for constructs that contain direct repeats to help reduce unwanted recombinants). After transformation, the small and large scale bacterial cultures were grown at 30°C. The lentiviral clones were verified using restriction digestion with both XhoI and AflII. An XhoI site is present in the lentiviral backbone at the 3' end of the insert. AflII sites are present within the lentiviral LTRs and at the 3' end of the gene of interest. The clones with the highest expression were packaged into the pseudotyped retrovirus. The 293FT (Invitrogen) packaging cell lines was co-transfected with 3 µg of lentiviral construct and 9 µg of ViraPower Packaging Mix (Invitrogen) with Lipofectamine 2000 (Invitrogen) in 10 cm dishes. The pLenti6.2/V5-DEST vector contains the human CMV immediate early promoter allowing high level constitutive expression of the gene of interest in most mammalian cells. The residual cellular debris was removed by 0.45µm syringe filter. The

supernatant containing the lentiviral particles was then ultracentrifuged in a Beckman 70.1 Ti rotor at 44,000rpm for 24 hours at 4°C on a 20% sucrose-TSE cushion [10mM Tris-Cl (pH 7.5), 100mM NaCl, 1mM EDTA]. The particles were resuspended in 600  $\mu$ L of TNE buffer [10mM Tris-Cl (pH 7.4), 100mM NaCl, 1mM EDTA]. Small aliquots (100  $\mu$ L) were stored at -80°C. The virus titer was performed by plating 293 HEK cells on 6-well plates (Grenier) at approximately 30-50% confluency. The next day, the lentiviral aliquots were diluted: mock,  $1.5 \times 10^{-4}$  and  $3 \times 10^{-4}$  in 1mL of complete culture medium and added to the 6-well plate. After 24 hours, the media was removed



**Figure A1. The ability of the pLenti6.2/V5-DEST HTLV-1 Tax to transactivate the NF $\kappa$ B-dependent transcription from the E-Selectin promoter-luciferase reporter plasmid** was demonstrated by cotransfecting 293 cells with 0.25  $\mu$ g of E-Selectin promoter-luciferase reporter plasmid and transduced with increasing amounts (150 $\mu$ L/mL and 300 $\mu$ L/mL) of unconcentrated pLenti6.2/V5-DEST HTLV-1 Tax or pLenti6.2/V5-DEST empty vector control. Relative luciferase activities were measured and normalized for equivalent total cellular protein levels. The data represent the mean  $\pm$  standard deviation (error bars) from three independent experiments. The expression of HTLV-1 Tax was detected by SDS-PAGE and immunoblotting.

and the cells were cultured in 2mL of complete media without virus overnight. On Day 4, the cells were harvested and detected by immunoblotting (Fig. A1.B). The viral supernatant was collected after 72 hrs and centrifuged at 2150 rpm for 7 minutes.

The pLenti6.2/V5-DEST HTLV-1 Tax was also used to study Tax-induced NF $\kappa$ B transactivation in 293 HEK cells using the E-Selectin promoter-luciferase reporter plasmid and was found to activate NF $\kappa$ B transcriptional activation (Fig. A1.A).



## APPENDIX B

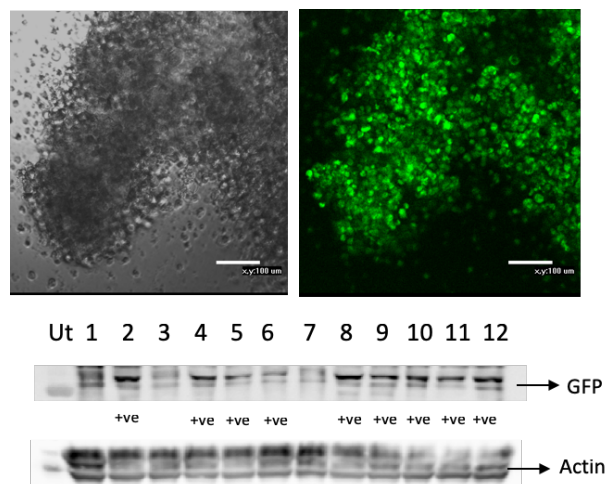
### **Generation of SLB1-GFP cell line**

The HTLV-1-transformed SLB1 lymphoma T-cells (kindly provided by P. Green, The Ohio State University-Comprehensive Cancer Center).  $2 \times 10^6$  cells were plated in a 10 cm tissue culture plate in IMDM supplemented with 10% FBS and antibiotics, in a humidified incubator at 37°C under 10% CO<sub>2</sub>. pLenti6.2V5-DEST HTLV-1 GFP virus was prepared by Romeo M (Romeo et al; 2018) and was used to transduce the SLB1 cells and were cultured for 48 hrs in media without blasticidin.

The pLenti6.2/V5-DEST vector backbone also contains a gene for resistance to the nucleoside antibiotic Blasticidin (Invitrogen). Blasticidin was used to remove any cells that were not transduced. A killing curve was used to determine the concentration of Blasticidin needed to kill non-transduced cells and allow for the propagation of stably transduced cells and ~5 µg/mL Blasticidin was determined to be optimal. The transduced SLB1-GFP cells were seeded in Poly L/Con A coated 96 well plates in 200µl complete media containing 5 µg/mL blasticidin and allowed to grow for 2 weeks. After 2 weeks, the 96 well plates were fed with 50µl complete media containing blasticidin and GFP detected through direct fluorescence.

The cells were left in culture for 2 more weeks in 96 well plates. After the 4<sup>th</sup> week as a result of blasticidin, the untransduced cells were eliminated, and the viable cell-wells were scaled up to 12 well plates with 500 µl complete media with 5 µg/mL blasticidin. The cells were allowed to grow in culture for 2 weeks after which they were fed 500 µl complete media with 2.5 µg/mL

blastocidin and allowed to grow for 2 more weeks. GFP was detected using direct fluorescence and viable wells were scaled up to 6 well cell culture plates in 1ml complete media with 1  $\mu$ g/mL blastocidin and allowed to grow for 1 week. The cells were scaled up to T-25 flasks and cultured in complete media with 1% blastocidin. After a week, the cells were pelleted by centrifugation at 2100 rpm at 4°C for 7 mins and the cell pellet were washed 4x with PBS. The cells were then resuspended in 7 ml IMDM supplemented with 10% FBS and antibiotics without blastocidin and cultured for 3 days. GFP was detected using direct fluorescence and 2 ml cells were harvested and lysed to get protein lysate. Immunoblotting was performed on the obtained lysate using rabbit polyclonal Anti-GFP (FL) antibody (Santacruz) and a rabbit secondary antibody (Jackson). Out of the 12 tested cell lines, 9 were positive for GFP on immunoblot detection (Fig. B1) and the remaining cells of these 9 cell lines were frozen in liquid nitrogen for future use.



**Figure B1. SLB1-GFP cell lines** GFP was detected through direct fluorescence. In the figure, DIC image (left) is shown and compared with green fluorescence (right) to show that over 90% cells had GFP expression in culture. SLB1-GFP cells were then harvested for protein lysates and immunoblotting was performed to detect the GFP protein. Relative Actin levels are shown by immunoblotting.

## REFERENCES

- Adya N, Zhao LJ, Huang W, Boros I, Giam CZ. (1994) Expansion of CREB's DNA recognition specificity by Tax results from interaction with Ala-Ala-Arg at positions 282-284 near the conserved DNA-binding domain of CREB. *Proc Natl Acad Sci USA* 91:5642-5646
- Akagi T, Ono H, Shimotohno K. (1995) Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1. *Blood* 86, 4243–4249
- Albrecht B, Collins ND, Burniston MT, Nisbet JW, Ratner L, Green PL, Lairmore MD. (2000) Human T-lymphotropic virus type 1 open reading frame I p12(I) is required for efficient viral infectivity in primary lymphocytes. *J. Virol.* 74:9828–9835.
- Alefantis T, Barmark K, Harhaj EW, Grant C, Wigdahl B. (2003) Characterization of a nuclear export signal within the human T-cell leukemia virus type I transactivator Tax. *J Biol Chem.* 278:21814–21822
- Andresen V, Pise-Masison C, Sinha-Datta U, Valeri VW, Parks RW, Cecchinato V, Fukumoto R, Nicot C, Franchini G. (2011) Suppression of HTLV-1 Replication by Tax-mediated rerouting of the p13 Viral Protein to Nuclear Speckles. *Blood* 118(6): 1549–1559
- Arima N, Molitor JA, Smith MR, Kim JH, Daitoku Y and Greene WC. (1991) Human T-cell leukemia virus type 1 Tax induces expression of the Rel-related family of KB enhancer-binding proteins: evidence for a pretranslational component of regulation. *J Virol.* 65:6892-6899
- Azran I, Schavinsky-Khrapunsky Y and Aboud M. (2004) Role of Tax protein in human T-cell leukemia virus type-I leukemogenicity. *Retrovirology* 1:20
- Bai XT, Baydoun HH, Nicot C. (2010) HTLV-I p30: A versatile protein modulating virus replication and pathogenesis. *Mol. Aspect. Med.* 31:344–349.
- Baldwin AS Jr. (2001) Series introduction: the transcription factor NF-kappaB and human disease. *J Clin Invest* 107:3-6.
- Ballard DW, Bohnlein E, Lowenthal JW, Wano Y, Franza BR, Greene WC. (1988) HTLV-I tax induces cellular proteins that activate the  $\kappa$ B element in the IL-2 receptor  $\alpha$  gene. *Science* 241:1652
- Baranger AM, Palmer CR, Hamm MK, Giebler HA, Brauweiler A, Nyborg JK, Schepartz A (1995) Mechanism of DNA-binding enhancement by the human T-cell leukaemia virus transactivator Tax. *Nature* 376:606-608

- Bartoe JT, Albrecht B, Collins ND, Robek MD, Ratner L, Green PL, Lairmore MD. (2000). Functional role of pX open reading frame II of human T-lymphotropic virus type 1 in maintenance of viral loads in vivo. *J. Virol.* 74:1094–1100.
- Baudhuin P. (1966) Lysosomes and cellular autophagy. *Brux Med.* 46:1059
- Baydoun HH, Bai XT, Shelton S, Nicot C. (2012) HTLV-I Tax Increases Genetic Instability by Inducing DNA Double Strand Breaks during DNA Replication and Switching Repair to NHEJ. *PLoS One.* 7(8):e42226
- Baydoun HH, Bellon M, Nicot C. (2008) HTLV-1 Yin and Yang: Rex and p30 master regulators of viral mRNA trafficking. *AIDS Rev.* 10:195–204.
- Baydoun HH, Pancewicz J, Nicot C. (2011) Human T-cell leukemia virus p30 inhibits homologous recombination and favors unfaithful DNA repair. *Blood* 10.1182
- Bazarbachi A, Plumelle Y, Carlos Ramos J, Tortevoe P, Otroek Z, Taylor G, Gessain A, Harrington W, Panelatti G, Hermine O. (2010) Meta-analysis on the use of zidovudine and interferon-alfa in adult T-cell leukemia/lymphoma showing improved survival in the leukemic subtypes. *J Clin Oncol.* 28(27):4177-4183
- Beilke MA, Traina-Dorge V, England JD, Blanchard JL. (1996) Polymyositis, arthritis, and uveitis in a macaque experimentally infected with human T lymphotropic virus type I. *Arthritis Rheum.* 39:610–615.
- Belletti B, Pellizzari I, Berton S, Fabris L, Wolf K, Lovat F, Schiappacassi M, D'Andrea S, Nicoloso MS, Lovisa S, Sonogo M, Defilippi P, Vecchione A, Colombatti A, Friedl P, Baldassarre G. (2010) p27kip1 controls cell morphology and motility by regulating microtubule-dependent lipid raft recycling. *Mol. Cell. Biol.* 30:2229-2240
- Bellon M, Nicot C. (2015) Multiple Pathways Control the Reactivation of Telomerase in HTLV-I-Associated Leukemia. *Int J Cancer Oncol.* 2(2): 215
- Bensaad K, Cheung EC, Vousden KH. (2009) Modulation of intracellular ROS levels by TIGAR controls autophagy. *EMBO J.* 28:3015–3026
- Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. *Cell* 126:107–120
- Bloom K. (2003) Microtubule cytoskeleton: navigating the intracellular landscape. *Curr Biol.* 13: R430-2
- Boxus M, Twizere JC, Legros S, Dewulf JF, Kettmann R, Willems L. (2008) The HTLV-1 Tax interactome. *Retrovirology* 14;5:76.
- Brooks C, Gu W. (2011) The impact of acetylation and deacetylation on the p53 pathway. *Protein Cell.* 2:456–462
- Cavanagh MH, Landry S, Audet B, Arpin-Andre C, Hivin P, Pare ME, Thete J, Wattel E, Marriott SJ, Mesnard JM, Barbeau B. (2006) HTLV-I antisense transcripts initiating in the 3'LTR are alternatively spliced and polyadenylated. *Retrovirology.* 3:15

- Cesarman E, Chadburn A, Inghirami G, Gaidano G, Knowles DM. (1992) Structural and functional analysis of oncogenes and tumor suppressor genes in adult T-cell leukemia/lymphoma shows frequent p53 mutations. *Blood* 80(12): 3205-3216
- Chaib-Mezrag H, Lemacon D, Fontaine H, Bellon M, Bai XT, Drac M, Coquelle A, Nicot C. (2014) Tax impairs DNA replication forks and increases DNA breaks in specific oncogenic genome regions. *Mol Cancer*. 13:205
- Chandhasin C, Ducu RI, Berkovich E, Kastan MB, Marriott SJ. (2008) Human T-cell leukemia virus type 1 tax attenuates the ATM-mediated cellular DNA damage response. *J. Virol.* 82:6952-6961
- Charbaut E, Curmi PA, Ozon S, Lachkar S, Redeker V, Sobel A. (2001) Stathmin family proteins display specific molecular and tubulin binding properties. *J Biol Chem*. 276:16146-54
- Ching YP, Chan SF, Jeang KT, Jin DY. (2006) The retroviral oncoprotein Tax targets the coiled-coil centrosomal protein TAX1BP2 to induce centrosome overduplication. *Nat. Cell Biol.*, 8:717-724
- Chlichlia K, Khazaie K. (2010) HTLV-1 Tax: Linking transformation, DNA damage and apoptotic T-cell death. *Chem Biol Interact*. 188(2):359-65
- Ciminale V, Pavlakis GN, Derse D, Cunningham CP, Felber BK. (1992) Complex splicing in the human T-cell leukemia virus (HTLV) family of retroviruses: novel mRNAs and proteins produced by HTLV type I. *J. Virol*. 66:1737-1745.
- Clerc I, Hivin P, Rubbo PA, Lemasson I, Barbeau B, Mesnard JM. (2009) Propensity for HBZ-SP1 isoform of HTLV-I to inhibit c-Jun activity correlates with sequestration of c-Jun into nuclear bodies rather than inhibition of its DNA-binding activity. *Virology* 391:195–202
- Clerc I, Polakowski N, Andre-Arpin C, Cook P, Barbeau B, Mesnard JM, Lemasson I (2008) An interaction between the human T cell leukemia virus type 1 basic leucine zipper factor (HBZ) and the KIX domain of p300/CBP contributes to the down-regulation of Tax-dependent viral transcription by HBZ. *J. Biol. Chem*. 283:23903
- Collins ND, Newbound GC, Albrecht B, Beard J, Ratner L, Lairmore MD. (1998) Selective ablation of human T-cell lymphotropic virus type 1 p12I reduces viral infectivity in vivo. *Blood* 91(12):4701–4707
- Cooper SA, Van der Loeff MS, Taylor GP. (2009) The neurology of HTLV-1 infection. *Pract. Neurol*. 9:16-26.
- Curmi PA, Gavet O, Charbaut E, Ozon S, Lachkae-Colmerauer S, Manceau V, Siavoshian S, MAucuer A, Sobel A. (1999) Stathmin and its phosphoprotein family: general properties, biochemical and functional interaction with tubulin. *Cell Struct Funct*. 24: 345-57
- Currer R, Van Duyne R, Jaworski E, Guendel I, Sampey G, Das R, Narayanan A, Kashanchi F. (2012) HTLV Tax: A Fascinating Multifunctional Co-Regulator of Viral and Cellular Pathways. *Front Microbiol*. 3: 406

- Dassouki Z, Sahin U, El Hajj H, Jollivet F, Kfoury Y, Lallemand-Breitenbach V, Hermine O, de Thé H, Bazarbachi A. (2015) ATL response to arsenic/interferon therapy is triggered by SUMO/PML/RNF4-dependent Tax degradation. *Blood*. 125(3):474-482.
- De Oliveira MFP, Vieira MG, Primo J, Siqueira IC, Carvalho EM, Farré L, Fatal PL, Bittencourt AL. (2010) Flower cells in patients with infective dermatitis associated with HTLV-1. *J Clin Virol* 48:288-90.
- DiDonato J, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, Karin M (1996) Mapping of the inducible I $\kappa$ B phosphorylation sites that signal its ubiquitination and degradation. *Mol. Cell. Biol.* 16:1295-1304 95.
- Ding W, Albrecht B, Luo R, Zhang W, Stanley JR, Newbound GC, Lairmore MD. (2001). Endoplasmic Reticulum and cis-Golgi Localization of Human T-Lymphotropic Virus Type 1 p12I: Association with Calreticulin and Calnexin. *J. Virol.* 75:7672–7682.
- Ding W, Kim SJ, Nair AM, Michael B, Boris-Lawrie K, Tripp A, Feuer G, Lairmore MD (2003) Human T-cell lymphotropic virus type 1 p12I enhances interleukin-2 production during T-cell activation. *J Virol.* 77(20):11027–11039.
- Dreux M, Chisari FV. (2009) Autophagy proteins promote hepatitis C virus replication. *Autophagy*. 5:1224–1225.
- Edwards D, Fenizia C, Gold H, de Castro-Amarante MF, Buchmann C, Pise-Masison CA, Franchini G. (2011) Orf-I and orf-II-encoded proteins in HTLV-1 infection and persistence. *Viruses* 3(6): 861-85.
- Fochi S, Mutascio S, Bertazzoni U, Zipeto D, Romanelli MG (2018) HTLV deregulation of the NF-kappaB pathway: an update on tax and antisense proteins role. *Front Microbiol.* 9:285
- Franchini G. (1995) Molecular mechanisms of human T-cell leukemia/lymphotropic virus type I infection. *Blood* 86(10):3619-3639
- Fukumoto R, Andresen V, Bialuk I, Cecchinato V, Walser JC, Valeri VW, Nauroth JM, Gessain A, Nicot C, Franchini G. (2009) In vivo genetic mutations define predominant functions of the human T-cell leukemia/lymphoma virus p12I protein. *Blood* 113: 3726–3734.
- Fukumoto R, Dunder M, Nicot C, Adams A, Valeri VW, Samelson LE, Franchini G. (2007) Inhibition of T-cell receptor signal transduction and viral expression by the linker for activation of T cells-interacting p12I protein of human T-cell leukemia/lymphoma virus type 1. *J Virol.* 81(17):9088–9099.
- Furukawa Y, Kubota R, Tara M, Izumo S, Osame M. (2001) Existence of escape mutant in HTLV-I tax during the development of adult T-cell leukemia. *Blood* 97, 987–993
- Gaudray G, Gachon F, Basbous J, Biard-Piechaczyk M, Devaux C, Mesnard JM. (2002) The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription. *J Virol.* 76:12813–12822
- Gessain A, Cassar O. (2012) Epidemiological Aspects and World Distribution of HTLV-1 Infection. *Frontiers in Microbiology.* 3:388

- Ghorbel S, Sinha-Datta U, Dundr M, Brown M, Franchini G, Nicot C. (2006) Human T-cell leukemia virus type I p30 nuclear/nucleolar retention is mediated through interactions with RNA and a constituent of the 60 S ribosomal subunit. *J. Biol. Chem.* 281:37150–37158
- Giebler HA, Loring JE, van Orden K, Colgin MA, Garrus JE, Escudero KW, Brauweiler A, Nyborg JK. (1997) Anchoring of CREB-binding protein to the human T-cell leukemia virus type 1 promoter: a molecular mechanism of Tax transactivation. *Mol. Cell. Biol.* 17:5156-5164
- Gonçalves DU, Proietti FA, Ramos Ribas JG, Araújo MG, Pinheiro SR, Guedes AC, Carneiro-Proietti ABF. (2010) Epidemiology, Treatment, and Prevention of Human T-Cell Leukemia Virus Type 1-Associated Diseases. *Clin Microbiol.* 23(3): 577–589
- Grassmann R, Dengler C, Muller-Fleckenstein I, Fleckenstein B, McGuire K, Dokhelar MC, Sodroski JG, Haseltine WA (1989) Transformation to continuous growth of primary human T lymphocytes by human T-cell leukemia virus type I X-region genes transduced by a Herpesvirus saimiri vector. *Proc. Natl. Acad. Sci. USA.* 86:3351-3355.
- Gröne M, Koch C, Grassmann R. (1996) The HTLV-1 Rex protein induces nuclear accumulation of unspliced viral RNA by avoiding intron excision and degradation. *Virology* 218 316–325
- Grossman WJ, Kimata JT, Wong FH, Zutter M, Ley TJ, Ratner L. (1995) Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I. *Proc Natl Acad Sci U S A.* 92(4):1057–1061
- Hagiya K, Yasunaga J, Satou Y, Ohshima K, Matsuoka M. (2011) ATF3, an HTLV-1 bZip factor binding protein, promotes proliferation of adult T-cell leukemia cells. *Retrovirology* 8-19
- Harhaj NS, Sun SC, Harhaj EW. (2007) Activation of NF- $\kappa$ B by the Human T Cell Leukemia Virus Type I Tax Oncoprotein Is Associated with Ubiquitin-dependent Relocalization of I $\kappa$ B Kinase. *J. Biol. Chem.* 282, 4185-4192
- Harrod R, Kuo YL, Tang Y, Yao Y, Vassilev A, Nakatani Y, Giam CZ. (2000) p300 and p300/cAMP-responsive element-binding protein associated factor interact with human T-cell lymphotropic virus type-1 Tax in a multi-histone acetyltransferase/activator-enhancer complex. *J Biol Chem* 275:11852-11857
- Harrod R, Tang Y, Nicot C, Lu HS, Vassilev A, Nakatani Y, Giam CZ. (1998) An exposed kid-like domain in human t-cell lymphotropic virus type 1 tax is responsible for the recruitment of coactivators cbp/p300. *Mol Cell Biol* 18:5052-5061
- Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sheehy N, Yamamoto Y, Ichinohe T, Tsunetsugu-Yokota Y, Katano H, Takahashi H, Matsuda J, Sata T, Kurata T, Nagashima K, Hall WW. (2006) Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I. *Nat Med.* 12(4):466–472
- Hatta Y, Koeffler HP. (2002) Role of tumor suppressor genes in the development of adult T cell leukemia/lymphoma (ATLL). *Leukemia* 16(6):1069-1085
- Hayden MS, Ghosh S. (2004) Signaling to NF- $\kappa$ B. *Genes Dev.* 18:2195-2224
- Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW, Vogelstein B (1997) 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol. Cell* 1:3-11

- Hidaka M, Inoue J, Yoshida M, Seiki M. (1988) Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins. *EMBO J.* 7;519–523
- Hinrichs SH, Nerenberg M, Reynolds RK, Khoury G, Jay G. (1987) A transgenic mouse model for human neurofibromatosis. *Science* 237:1340-1343.
- Hiraragi H, Michael B, Nair A, Silic-Benussi M, Ciminale V, Lairmore M. (2005) Human T-lymphotropic virus type 1 mitochondrion-localizing protein p13II sensitizes Jurkat T cells to Ras-mediated apoptosis. *J. Virol.* 79:9449–9457
- Hiscott J, Kwon H, Génin P. (2001) Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J Clin Invest.* 107(2):143-51
- Hivin P, Basbous J, Raymond F, Henaff D, Arpin-Andre C, Robert-Hebmann V, Barbeau B, Mesnard JM. (2007) The HBZ-SP1 isoform of human T-cell leukemia virus type I represses JunB activity by sequestration into nuclear bodies. *Retrovirology* 4:14
- Hivin P, Frederic M, Arpin-Andre C, Basbous J, Gay B, Thebault S, Mesnard JM. (2005) Nuclear localization of HTLV-I bZIP factor (HBZ) is mediated by three distinct motifs. *J Cell Sci.* 118:1355–1362
- Holden NS, Squires PE, Kaur M, Bland R, Jones CE, Newton R. (2008) Phorbol ester-stimulated NF-kappaB-dependent transcription: roles for isoforms of novel protein kinase C. *Cell Signal.* 20(7):1338-48
- Hong S, Wang LC, Gao X, Kuo YL, Liu B, Merling R, Kung HJ, Shih HM, and Giam CZ. (2007) Heptad repeats regulate protein phosphatase 2a recruitment to I-kappaB kinase gamma/NF-kappaB essential modulator and are targeted by human T-lymphotropic virus type 1 Tax. *J. Biol. Chem.* 282:12119-12126
- Howes SC, Alushin GM, Shida T, Nachury MV, Nogales E. (2014) Effects of tubulin acetylation and tubulin acetyltransferase binding on microtubule structure. *Mol. Biol. Cell* 25:257- 266
- Huang J, Ren T, Guan H, Jiang Y, Cheng H. (2009) HTLV-1 Tax Is a Critical Lipid Raft Modulator That Hijacks IκB Kinases to the Microdomains for Persistent Activation of NF-κB. *J. Biol. Chem.* 284, 6208-6217
- Igakura T, Stinchcombe JC, Goon PK, Taylor GP, Weber JN, Griffiths GM, Tanaka Y, Osame M, Bangham CR. (2003) Spread of HTLV-I between lymphocytes by virus-induced polarization of the cytoskeleton. *Science* 299, 1713–1716
- Inoue J, Yoshida M, Seiki M. (1987) Transcriptional (p40x) and post-transcriptional (p27x-III) regulators are required for the expression and replication of human T-cell leukemia virus type I genes. *Proc Natl Acad Sci U S A.* 84(11):3653-7
- Ishihara S, Tachibana N, Okayama A, Murai K, Tsuda K, Mueller N. (1992) Successful graft of HTLV-I-transformed human T-cells (MT-2) in severe combined immunodeficiency mice treated with anti-asialo GM-1 antibody. *Jpn J Cancer Res.* 83(4):320–323
- Iwakura Y, Saijo S, Kioka Y, Nakayama-Yamada J, Itagaki K, Tosu M, Asano M, Kanai Y, Kakimoto K. (1995) Autoimmunity induction by human T cell leukemia virus type 1 in



- transgenic mice that develop chronic inflammatory arthropathy resembling rheumatoid arthritis in humans. *J Immunology*. 155(3):1588–1598
- Jeang KT, Giam CZ, Majone F, Aboud M. (2004) Life, Death, and Tax: Role of HTLV-I Oncoprotein in Genetic Instability and Cellular Transformation. *J Biol Chem*. 279(31):31991-4
- Jeang KT, Widen SG, Semmes OJ, Wilson SH. (1990) HTLV-I trans-activator protein, Tax, is a trans-repressor of the human  $\beta$ -polymerase gene. *Science* 247:1082–1084
- Jeang KT. (2001) Functional activities of the human T-cell leukemia virus type I Tax oncoprotein: cellular signaling through NF-kappa B. *Cytokine Growth Factor Rev*. 12;207–217.
- Jeong SJ, Radonovich M, Brady J, Pise-Masison CA. (2004) HTLV-I Tax induces a novel interaction between p65/RelA and p53 that results in inhibition of p53 transcriptional activity. *Blood*. 104:1490–1497
- Jin DY, Jeang KT. (1997) HTLV-I Tax self-association in optimal trans-activation function. *Nucl Acids Res*. 25:379–387
- Jin DY, Spencer F, Jeang KT (1998) Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell*. 93:81–91.
- Johnson JM, Nicot C, Fullen J, Ciminale V, Casareto L, Mulloy JC, Jacobson S, Franchini G. (2001) Free major histocompatibility complex class I heavy chain is preferentially targeted for degradation by human T-cell leukemia/lymphotropic virus type 1 p12(I) protein. *J Virol*. 75(13):6086–6094.
- Jones KS, Petrow-Sadowski C, Huang YK, Bertolette DC, Ruscetti FW. (2008) Cell- free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4+ T cells. *Nat Med*. 14(4): 429-436
- Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. (2003) Microtubule disruption utilizes an NFkappa B-dependent pathway to stabilize HIF-1alpha protein. *J Biol Chem*. 278:7445- 52.
- Kamada N, Sakurai M, Miyamoto K, Sanada I, Sadamori N, Fukuhara S, Abe S, Shiraishi Y, Abe T, Kaneko Y, Shimoyama M (1992) Chromosome abnormalities in adult T-cell leukemia/lymphoma: a Karyotype Review Committee report. *Cancer Research*. 52;1481–1493
- Kao S-Y, Marriott SJ. (1999) Disruption of nucleotide excision repair by the human T-cell leukemia virus type 1 Tax protein. *J Virol* 73:4299– 4304
- Kasai T, Iwanaga Y, Iha H, Jeang KT. (2002) Prevalent Loss of Mitotic Spindle Checkpoint in Adult T-cell Leukemia Confers Resistance to Microtubule Inhibitors *J. Biol. Chem*. 277:5187–5193
- Kazanji M, Ureta-Vidal A, Ozden S, Tangy F, de Thoisy B, Fiette L, Talarmin A, Gessain A, The G. (2000) Lymphoid Organs as a Major Reservoir for Human T-Cell Leukemia Virus Type 1 in Experimentally Infected Squirrel Monkeys (*Saimiri sciureus*): Provirus Expression, Persistence, and Humoral and Cellular Immune Responses. *Journal of Virology*. 74(10):4860-4867

- Kchour G, Tarhini M, Kooshyar MM, El Hajj H, Wattel E, Mahmoudi M, Hatoum H, Rahimi H, Maleki M, Rafatpanah H, Rezaee SA, Yazdi MT, Shirdel A, de Thé H, Hermine O, Farid R, Bazarbachi A. (2009) Phase 2 study of the efficacy and safety of the combination of arsenic trioxide, interferon alpha, and zidovudine in newly diagnosed chronic adult T-cell leukemia/lymphoma(ATL). *Blood*. 113(26):6528-6532
- Kehn K, Fuente Cde L, Strouss K, Berro R, Jiang H, Brady J, Mahieux R, Pumfery A, Bottazzi ME, Kashanchi F. (2005) The HTLV-I Tax oncoprotein targets the retinoblastoma protein for proteasomal degradation. *Oncogene*. 24:525–540
- Kim JY, Wang L, Lee J, Ou JJ. (2017) HCV induces the localization of lipid rafts to autophagosomes for its RNA replication. *J Virol*. 91(20)
- Kimata JT, Wong FH, Wang JJ, Ratner L. (1994) Construction and characterization of infectious human T-cell leukemia virus type I molecular clones. *Virology* 204(2):656–664.
- Koiwa T, Hamano-Usami A, Ishida T, Okayama A, Yamaguchi K, Kamihira S, Watanabe T. (2002) 5'-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo. *J. Virol*. 76, 9389–9397
- Koralnik IJ, Fullen J, Franchini G. (1993) The p12I, p13II, and p30II proteins encoded by human T-cell leukemia/lymphotropic virus type I open reading frames I and II are localized in three different cellular compartments. *J Virol*. 67(4):2360–2366
- Kotani S, Yoshimoto S, Yamato K, Fujishita M, Yamashita M, Ohtsuki Y, Taguchi H, Miyoshi I. (1986) Serial transmission of human T-cell leukemia virus type I by blood transfusion in rabbits and its prevention by use of X-irradiated stored blood. *Int J Cancer*. 37(6):843–847
- Kuhlmann AS, Villaudy J, Gazzolo L, Castellazzi M, Mesnard JM, Duc Dodon M. (2007) HTLV-1 HBZ cooperates with JunD to enhance transcription of the human telomerase reverse transcriptase gene (hTERT) *Retrovirology* 4-92
- La Grenade L, Hanchard B, Fletcher V, Cranston B, Blattner W. (1990) Infective dermatitis of Jamaican children: a marker of HTLV-I infection. *Lancet* 336:1345-1347
- Lairmore MD, Silverman L, Ratner L. (2005) Animal models for human T-lymphotropic virus type 1 (HTLV-1) infection and transformation. *Oncogene* 24(39):6005-6015
- Ledoux AC, Sellier H, Gillies K, Iannetti A, James J, Perkins ND. (2013) NF- $\kappa$ B regulates expression of Polo-like kinase 4. *Cell Cycle* 12:3052-3062.
- Lee DY, Sugden B. (2008) The latent membrane protein 1 oncogene modifies B-cell physiology by regulating autophagy. *Oncogene*. 27:2833–2842
- Lee TH, Coligan JE, Homma T, McLane MF, Tachibana N, Essex M. (1984) Human T-cell leukemia virus-associated membrane antigens (HTLV-MA): Identity of the major antigens recognized after virus infection. *Proc Natl Acad Sci U S A*. 81:3856–3860.
- Lefebvre L, Vanderplasschen A, Ciminale V, Heremans H, Dangoisse O, Jauniaux JC, Toussaint JF, Zelnik V, Burny A, Kettmann R and Willems L. (2002) Oncoviral Bovine Leukemia Virus G4 and Human T-Cell Leukemia Virus Type 1 p13II Accessory Proteins Interact with Farnesyl Pyrophosphate Synthetase. *J. Virol*. 76:1400–1414

- Lemoine FJ, Kao S-Y, Marriott SJ. (2000) Suppression of DNA repair by HTLV type 1 Tax correlates with Tax trans-activation of proliferating cell nuclear antigen gene expression. *AIDS Res Hum Retroviruses* 16:1623–1627
- Li H, Jogl G (2009) Structural and biochemical studies of TIGAR (TP53-induced glycolysis and apoptosis regulator). *J Biol Chem.* 284:1748–1754
- Liu B, Hong S, Tang Z, Yu H, Giam CZ. (2005) HTLV-I Tax directly binds the Cdc20-associated anaphase-promoting complex and activates it ahead of schedule. *Proc. Natl. Acad. Sci USA.* 102:63–68
- Liu B, Liang MH, Kuo YL, Liao W, Boros I, Kleinberger T, Blancato J, Giam CZ. (2003). Human T-lymphotropic virus type 1 oncoprotein tax promotes unscheduled degradation of Pds1p/securin and Clb2p/cyclin B1 and causes chromosomal instability. *Mol. Cell. Biol.* 23:5269–5281
- Loeb KR, Loeb LA. (2000) Significance of multiple mutations in cancer. *Carcinogenesis.* 21:379–385
- Low KG, Dorner LF, Fernando DB, Grossman J, Jeang KT, Comb MJ. (1997) Human T-cell leukemia virus type 1 Tax releases cell cycle arrest induced by p16INK4a. *J. Virol.* 71:1956–1962.
- Lundblad JR, Kwok RP, Laurance ME, Huang MS, Richards JP, Brennan RG, Goodman RH. (1998) The human T-cell leukemia virus-1 transcriptional activator Tax enhances cAMP-responsive element-binding protein (CREB) binding activity through interactions with the DNA minor groove. *J Biol Chem.* 273:19251-19259
- Ma G, Yasunaga J, Fan J, Yanagawa S, Matsuoka M. (2013) HTLV-1 bZIP factor dysregulates the Wnt pathways to support proliferation and migration of adult T-cell leukemia cells. *Oncogene* 32:4222
- Ma Y, Zheng S, Wang Y, Zang W, Li M, Wang N, Li P, Jin J, Dong Z, Zhao G. (2013) The HTLV-1 HBZ protein inhibits cyclin D1 expression through interacting with the cellular transcription factor CREB. *Mol. Biol. Rep.* 40:5967–5975
- Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H, Wirth T. (2010) NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. *Cancer Lett* 295:214-228
- Manel N, Kim FJ, Kinet S, Taylor N, Sitbon M, Battini JL. (2003) The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV. *Cell* 115, 449–459
- Manns A, Hisada M, La Grenade L. (1999) Human T-lymphotropic virus type I infection. *Lancet.* 353(9168):1951-8
- Marriott SJ, Lemoine FJ, Jeang KT. (2002) Damaged DNA and miscounted chromosomes: human T cell leukemia virus type I tax oncoprotein and genetic lesions in transformed cells. *J Biomed Sci.* 9(4):292-8

- Matarrese P, Garofalo T, Manganelli V, Gambardella L, Marconi M, Grasso M, Tinari A, Misasi R, Malorni W, Sorice M. (2014) Evidence for the involvement of GD3 ganglioside in autophagosome formation and maturation. *Autophagy*. 10:750-65
- Matsuoka M, Jeang KT. (2005) Human T-cell leukemia virus type I at age 25: a progress report. *Cancer Res*. 65(11):4467–70
- Matsuoka M. (2003) Human T-cell leukemia/lymphoma virus type 1 and adult T-cell leukemia. *Oncogene* 22(33): 5131-40
- Matsuoka M, Green PL (2009) The HBZ gene, a key player in HTLV-1 pathogenesis. *Retrovirology*. 6-71.
- McGinn TM, Tao B, Cartner S, Schoeb T, Davis I, Ratner L, Fultz PN. (2002) Association of Primate T-Cell Lymphotropic Virus Infection of Pig-Tailed Macaques with High Mortality. *Virology*. 304:364–378
- Michael B, Nair AM, Datta A, Hiraragi H, Ratner L, Lairmore MD. (2006) Histone acetyltransferase (HAT) activity of p300 modulates human T lymphotropic virus type 1 p30II-mediated repression of LTR transcriptional activity. *Virology* 354:225–239
- Mitobe Y, Yasunaga J, Furuta R, Matsuoka M. (2015) HTLV-1 bZIP Factor RNA and Protein Impart Distinct Functions on T-cell Proliferation and Survival. *Cancer Res*. 75(19):4143-52
- Miyake H, Suzuki T, Hirai H, Yoshida M. (1999) Trans-activator Tax of Human T-Cell Leukemia Virus Type 1 Enhances Mutation Frequency of the Cellular Genome *Virology* 253, 155–161
- Miyoshi I, Yoshimoto S, Kubonishi I, Fujishita M, Ohtsuki Y, Yamashita M, Yamato K, Hirose S, Taguchi H, Niiya K, Kobayashi M. (1985) Infectious transmission of human T-cell leukemia virus to rabbits. *Int J Cancer*. 35(1):81–85
- Mochizuki M, Watanabe T, Yamaguchi K, Yoshimura K, Nakashima S, Shirao M, Araki S, Takatsuki K, Mori S, Miyata N. (1992) Uveitis associated with human T-cell lymphotropic virus type I: seroepidemiologic, clinical and virologic studies. *J. Infect. Dis*. 166:943-944
- Mori N, Sato H, Hayashibara T, Senba M, Hayashi T, Yamada Y, Kamihira S, Ikeda S, Yamasaki Y, Morikawa S, Tomonaga M, Geleziunas R, Yamamoto N (2002) Human T-cell leukemia virus type I Tax transactivates the matrix metalloproteinase-9 gene: potential role in mediating adult T-cell leukemia invasiveness. *Blood*. 99:1341–1349
- Mortreux F, Kazanji M, Gabet A-S, de Thoisy B, Wattel E. (2001) Two-Step Nature of Human T-Cell Leukemia Virus Type 1 Replication in Experimentally Infected Squirrel Monkeys (*Saimiri sciureus*). *Journal of Virology* 75(2):1083-1089
- Nakagawa MK, Nakahara Y, Maruyama M, Kawabata I, Higuchi I, Kubota H, Izumo S, Arimura K, Osame M. (1996) Therapeutic trials in 200 patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. *J. Neurovirol*. 2:345-355
- Nejmeddine M, Barnard AL, Tanaka Y, Taylor GP, Bangham C. (2005) Human T-lymphotropic Virus, Type 1, Tax Protein Triggers Microtubule Reorientation in the Virological Synapse. *J. Biol. Chem*. 280, 29653-29660

- Nerenberg M, Hinrichs SM, Reynolds RK, Khoury G, Jay G. (1987) The tat gene of human T-lymphotrophic virus type I induces mesenchymal tumors in transgenic mice. *Science*. 237(4820):1324–1329
- Nerenberg MI. (1990) An HTLV-I Transgenic Mouse Model: Role of the tax gene in pathogenesis in multiple organ systems. *Curr. Top. Microbiol. Immunol.* 160:121–128
- Nicot C, Mulloy JC, Ferrari MG, Johnson JM, Fu K, Fukumoto R, Trovato R, Fullen J, Leonard WJ, Franchini G. HTLV-1 p12(I) protein enhances STAT5 activation and decreases the interleukin-2 requirement for proliferation of primary human peripheral blood mononuclear cells. *Blood*. 2001;98(3):823–829.
- Nicot C., Dunder M., Johnson J.M., Fullen J.R., Alonzo N., Fukumoto R., Princler G.L., Derse D., Misteli T., Franchini G. (2004) HTLV-1-encoded p30II is a post-transcriptional negative regulator of viral replication. *Nat. Med.* 10:197–201
- Nyborg JK, Egan D, Sharma N. (2010) The HTLV-1 Tax protein: revealing mechanisms of transcriptional activation through histone acetylation and nucleosome disassembly. *Biochim. Biophys. Acta* 1799, 266–274.
- Ohashi T, Hanabuchi S, Kato H, Koya Y, Takemura F, Hirokawa K, Yoshiki T, Tanaka Y, Fujii M, Kannagi M. (1999) Prevention of Adult T-Cell Leukemia-Like Lymphoproliferative Disease in Rats by Adoptively Transferred T Cells from a Donor Immunized with Human T-Cell Leukemia Virus Type 1 Tax-Coding DNA Vaccine. *J Virol.* 73:6031–6040
- Ohsugi T, Yamaguchi K, Kumasaka T, Ishida T, Horie R, Watanabe T, Sakio N, Fujimoto T, Sakamoto N, Urano T. (2004) Rapid tumor death model for evaluation of new therapeutic agents for adult T-cell leukemia. *Lab Invest.* 84(2):263–266
- Ohtani K, Iwanaga R, Arai M, Huang Y, Matsumura Y, Nakamura M. (2000) Cell type-specific E2F activation and cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I. *J Biol Chem* 275:11154–11163
- Osame M, Matsumoto M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A (1987) Chronic progressive myelopathy associated with elevated antibodies to human T-lymphotropic virus type I and adult T-cell leukemia like cells. *Ann. Neurol.* 21:117–122.
- Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, et al. (1986) HTLV-I associated myelopathy, a new clinical entity. *Lancet.* 1(8488):1031-2
- Panfil AR, Al-Saleem JJ, Green PL. (2013) Animal Models Utilized in HTLV-1 Research. *Virology (Auckl).* 4:49–59
- Peloponese Jr. JM, Haller K, Miyazato A, Jeang KT. (2005) Abnormal centrosome amplification in cells through the targeting of Ran-binding protein-1 by the human T cell leukemia virus type-1 Tax oncoprotein. *Proc. Natl. Acad. Sci. U.S.A.* 102:18974-18979
- Peloponese Jr. JM, Kinjo T, Jeang KT. (2007) Human T-cell leukemia virus type 1 Tax and cellular transformation. *Int. J Hematol* 86(2):101-6
- Perini G, Wagner S, Green MR (1995) Recognition of bZIP proteins by the human T-cell leukaemia virus transactivator Tax. *Nature* 376:602-605

- Philip S., Zahoor M.A., Zhi H., Ho Y.K., Giam C.Z. (2014) Regulation of human T-lymphotropic virus type I latency and reactivation by HBZ and Rex. *PLoS Pathog.* 10:34
- Pise-Masison C.A. and Brady J.N. (2005) Setting the stage for transformation: HTLV-1 Tax inhibition of p53 function. *Front. Biosci.* 10:919-930.
- Pise-Masison CA, Choi KS, Radonovich M, Dittmer J, Kim SJ, Brady JN. (1998) Inhibition of p53 transactivation function by the human T-cell lymphotropic virus type 1 Tax protein. *J. Virol.* 72:1165-1170
- Pise-Masison CA, Mahieux R, Jiang H, Ashcroft M, Radonovich M, Duvall J, Guillerme C, Brady J. (2000) Inactivation of p53 by human T-cell lymphotropic virus type 1 Tax requires activation of the NF-kappaB pathway and is dependent on p53 phosphorylation. *Mol. Cell. Biol.* 20:3377-3386.
- Pise-Masison CA, Mahieux R, Radonovich M, Jiang H, Duvall J, Guillerme C, Brady JN. (2000) Insights into the molecular mechanism of p53 inhibition by HTLV type 1 Tax. *AIDS Res. Hum. Retroviruses* 16:1669-1675
- Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. (1980) Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA.* 77(12):7415-9
- Pozzatti R, Vogel J, Jay G. (1990) The human T-lymphotropic virus type I tax gene can cooperate with the ras oncogene to induce neoplastic transformation of cells. *Mol. Cell. Biol.* 10:413-417.
- Proietti FA, Carneiro-Proietti ABF, Catalan-Soares BC, Murphy EL. (2005) Global epidemiology of HTLV-1 infection and associated diseases. *Oncogene* 24:6058-6068
- Qu Z, Xiao G. (2011) Human T-cell lymphotropic virus: a model of NF-κB-associated tumorigenesis. *Viruses* 3, 714-749
- Rana S, Maples PB, Senzer N, Nemunaitis J. (2008) Stathmin 1: a novel therapeutic target for anticancer activity. *Expert Rev Anticancer Ther.* 8:1461-70
- Raunch DA, Ratner L. (2011) Targeting HTLV-1 activation of NFκB in mouse models and ATLL patients. *Viruses* 3(6):886-900
- Ren T, Takahashi Y, Liu X, Loughran TP, Sun SC, Wang HG, Cheng H. (2015) HTLV-1 Tax deregulates autophagy by recruiting autophagic molecules into lipid raft microdomains. *Oncogene.* 34(3):334-345
- Rice NR, MacKichan ML, Israel A. (1992) The precursor of NF-kappa B p50 has I kappa B-like functions. *Cell.* 71:243-253
- Rivera-Walsh I, Waterfield M, Xiao G, Fong A, Sun SC. (2001) NF-kappaB signaling pathway governs TRAIL gene expression and human T-cell leukemia virus-I Tax-induced T-cell death. *J. Biol. Chem.* 276:40385-40388

- Robek M, Ratner L. (1999) Immortalization of CD4(+) and CD8(+) T lymphocytes by human T-cell leukemia virus type 1 Tax mutants expressed in a functional molecular clone. *J. Virol.* 73:4856–4865.
- Rocks O, Peyker A, Kahms M, Verveer PJ, Koerner C, Lumbierres M, Kuhlmann J, Waldmann H, Wittinghofer A, Bastiaens PI. (2005) *Science.* 307:1746-1752
- Saggioro D, Silic-Benussi M, Biasiotto R, D’Agostino DM, Ciminale V. (2009) Control of cell death pathways by HTLV-1 proteins. *Front. Biosci.* 14:3338–3351
- Saito M, Matsuzaki T, Satou Y, Yasunaga J, Saito K, Arimura K, Matsuoka M, Ohara Y. (2009) In vivo expression of the HBZ gene of HTLV-1 correlates with proviral load, inflammatory markers and disease severity in HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP). *Retrovirology.* 6
- Saitoh Y, Yamamoto N, Dewan MZ, Sugimoto H, Martinez Bruyn VJ, Iwasaki Y, Matsubara K, Qi X, Saitoh T, Imoto I, Inazawa J, Utsonomiya A, Watanabe T, Masuda T, Yamamoto N, Yamaoka S. (2008) Overexpressed NF- $\kappa$ B-inducing kinase contributes to the tumorigenesis of adult T-cell leukemia and Hodgkin Reed-Sternberg cells. *Blood* 111:5118–5129
- Satou Y, Nosaka K, Koya Y, Yasunaga JI, Toyokuni S, Matsuoka M. (2004) Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro. *Leukemia.* 18:1357–1363
- Schiappacassi M, Lovisa S, Lovat F, Fabris L, Colombatti A, Belletti B, Baldassarre G. (2011) Role of T198 modification in the regulation of p27(Kip1) protein stability and function. *PLoS One.* 6:e17673
- Schmitt I, Rosin O, Rohwer P, Gossen M, Grassmann R. (1998) Stimulation of cyclin-dependent kinase activity and G1- to S-phase transition in human lymphocytes by the human T-cell leukemia/lymphotropic virus type 1 Tax protein. *J Virol* 72:633-640
- Shibata Y, Tokunaga F, Goto E, Komatsu G, Gohda J, Saeki Y, Tanaka K, Takahashi H, Sawasaki T, Inoue S, Oshiumi H, Seya T, Nakano H, Tanaka Y, Iwai K, Inoue J. (2017) HTLV-1 Tax induces formation of the active macromolecular IKK complex by generating Lys63- and Met1-linked hybrid polyubiquitin chains. *PLoS Pathog.* 13(1): e1006162
- Shimoyama M. (1991) Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87). *British Journal of Haematology.* 79:428–437
- Shirono K, Hattori T, Takatsuki K. (1994) A new classification of clinical stages of adult T-cell leukemia based on prognosis of the disease. *Leukemia.* 8(11):1834-1837
- Silic-Benussi M., Marin O., Biasiotto R., D’Agostino D. M., Ciminale V. (2010) Effects of human T-cell leukemia virus type 1 (HTLV-1) p13 on mitochondrial K<sup>+</sup> permeability: a new member of the viroporin family? *FEBS Lett.* 584 2070–2075
- Silverman LR, Phipps AJ, Montgomery A, Ratner L and Lairmore MD. (2004) Human T-cell lymphotropic virus type 1 open reading frame II-encoded p30II is required for in vivo replication: evidence of in vivo reversion. *J. Virol.* 78:3837–3845

- Silverman LR, Phipps AJ, Montgomery A, Ratner L, Lairmore MD. (2004) Human T-cell lymphotropic virus type 1 open reading frame II-encoded p30II is required for in vivo replication: evidence of in vivo reversion. *J Virol.* 78(8):3837–3845
- Silverman N, and Maniatis T. (2001). NF-kappaB signaling pathways in mammalian and insect innate immunity. *Genes Dev.* 15, 2321–2342
- Sinha-Datta U, Datta A, Ghorbel S, Dodon MD, Nicot C. (2007) Human T-cell lymphotropic virus type I rex and p30 interactions govern the switch between virus latency and replication. *J. Biol. Chem.* 282:14608–14615.
- Smith MR, Greene WC. (1991) Molecular biology of type I human T-cell leukemia virus (HTLV-I) and adult T-cell leukemia. *J Clin Invest.* 87:761
- Smith MR, Greene WC. (1992) Characterization of a novel nuclear localization signal in the HTLV-I tax transactivator protein. *Virology* 187:316–320
- Sorice M, Matarrese P, Manganelli V, Tinari A, Giammarioli AM, Mattei V, Misasi R, Garofalo T, Malorni W. (2010) Role of GD3-CLIPR-59 association in lymphoblastoid T cell apoptosis triggered by CD95/Fas. *Plos One.* 5:e8567
- Suga T, Kameyama T, Kinoshita T, Shimotohno K, Matsumura M, Tanaka H, Kushida S, Ami Y, Uchida M, Uchida K, Miwa M. (1991) Infection of rats with HTLV-I: a small-animal model for HTLV-I carriers. *Int J Cancer.* 49(5):764–769
- Sun SC and Ballard DW. (1999) Persistent activation of NF- $\kappa$ B by the Tax transforming protein of HTLV-1: hijacking cellular I $\kappa$ B kinases. *Oncogene* 18:6948–6958
- Sun SC. (2011). Non-canonical NF-kappaB signaling pathway. *Cell Res.* 21, 71–85
- Sykes SM, Mellert HS, Holbert MA, Li K, Marmorstein R, Lane WS, McMahon SB. (2006) Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol. Cell* 24, 841–851
- Sylla, BS, Hung SC, Davidson DM, Hatzivassiliou E, Malinin NL, Wallach D, Gilmore TD, Kieff E, Mosialos G. (1998) Epstein-Barr virus-transforming protein latent infection membrane protein 1 activates transcription factor NF- $\kappa$ B through a pathway that includes the NF- $\kappa$ B-inducing kinase and the I $\kappa$ B kinases IKK $\alpha$  and IKK $\beta$ . *Proc. Natl. Acad. Sci., USA.* 95:10106–10111
- Takatsuki K, Yamaguchi K, Kawano F, Hattori T, Nishimura H, Tsuda H, Sanada I, Nakada K, Itai Y. (1985) Clinical Diversity in Adult T-Cell Leukemia-Lymphoma. *Cancer Research (Suppl.)*. 45:4644s–4645s
- Takeda S, Maeda M, Morikawa S, Taniguchi Y, Yasunaga J, Nosaka K, Tanaka Y, Matsuoka M. (2004) Genetic and epigenetic inactivation of tax gene in adult T-cell leukemia cells. *Int. J. Cancer* 109:559–567
- Takenouchi N, Jones KS, Lisinski I, Fugo K, Yao K, Cushman SW, Ruscetti FW, Jacobson S. (2007) GLUT1 Is Not the Primary Binding Receptor but Is Associated with Cell-to-Cell Transmission of Human T-Cell Leukemia Virus Type 1. *J. Virol.* 81:1506–1510



- Tamiya S, Matsuoka M, Etoh K, Watanabe T, Kamihira S, Yamaguchi K, Takatsuki K. (1996) Two types of defective human T-lymphotropic virus type I provirus in adult T-cell leukemia. *Blood* 88, 3065–3073
- Tanaka A, Takahashi C, Yamaoka S, Nosaka Y, Maki M, Hatanaka M. (1990) Oncogenic transformation by the tax gene of human T-cell leukemia virus type I in vitro. *Proc. Natl. Acad. Sci. USA.* 87:1071-1075.
- Tanaka Y, Hamano S, Gotoh K, Murata Y, Kunisaki Y, Nishikimi A, Takii R, Kawaguchi M, Inayoshi A, Masuko S, Himeno K, Sasazuki T, Fukui Y. (2007) T helper type 2 differentiation and intracellular trafficking of the interleukin 4 receptor- $\alpha$  subunit controlled by the Rac activator Dock2. *Nat. Immunol.* 8:1067–1075.
- Tanaka-Nakanishi A., Yasunaga J., Takai K., Matsuoka M. (2014) HTLV-1 bZIP factor suppresses apoptosis by attenuating the function of FoxO3a and altering its localization. *Cancer Res.* 74:188–200
- Tang H, Da L, Mao Y, Li Y, Li D, Xu Z, Li F, Wang Y, Tiollais P, Li T, Zhao M. (2009) Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy via up-regulation of beclin 1 expression. *Hepatology.* 49:60–71.
- Tang Y, Luo J, Zhang W, Gu W. (2006) Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol. Cell* 24, 827-839
- Tang Y, Kwon H, Neel BA, Kasher-Meron M, Pessin J, Yamada E, Pessin J. (2018) The fructose-2,6-bisphosphatase TIGAR suppresses NF- $\kappa$ B signaling by directly inhibiting the linear ubiquitin assembly complex LUBAC. *J. Biol. Chem.* 293, 7578-7591
- Taniguchi Y, Nosaka K, Yasunaga J, Maeda M, Mueller N, Okayama A, Matsuoka M. (2005) Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms. *Retrovirology* 2:64
- Taylor G. (2007) Molecular aspects of HTLV-I infection and adult T-cell leukaemia/lymphoma. *J Clin Pathol.* 60(12):1392-6
- Taylor GP and Matsuoka M. (2005) Natural history of adult T-cell leukemia/lymphoma and approaches to therapy. *Oncogene* 24(39): 6047-57
- Tsukasaki K, Watanabe T, Tobinai K. (2013) Adult T-cell leukemia–lymphoma. *Abeloff’s clinical oncology.* 5. Philadelphia: Elsevier Saunders; 2076–2092.
- Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. (1977) Adult T-cell leukemia: clinical and hematologic features of 16 cases. *Blood.* 50:481–492
- Utsunomiya A, Choi I, Chihara D, Seto M. (2015) Recent advances in the treatment of adult T-cell leukemia-lymphomas. *Cancer Sci.* 106(4):344-351
- Van Prooyen N, Andresen V, Gold H, Bialuk I, Pise-Masison C, Franchini G. (2010) Hijacking the T-cell communication network by the human T-cell leukemia/lymphoma virus type 1 (HTLV-1) p12 and p8 proteins. *Mol Aspects Med* 31(5): 333-43.

- Van Prooyen N, Gold H, Andresen V, Schwartz O, Jones K, Ruscetti F, Lockett S, Gudla P, Venzon D, Franchini G. (2010) Human T-cell leukemia virus type 1 p8 protein increases cellular conduits and virus transmission. *Proc Natl Acad Sci USA*. 107: 20738–20743.
- Verdonck K, González E, Van Dooren S, Vandamme AM, Vanham G, Gotuzzo E. (2007) Human T-lymphotropic virus 1: recent knowledge about an ancient infection. *Lancet Infect Dis*. 7(4):266-81
- Wang J, Jacob NK, Ladner KJ, Beg A, Perko JD, Tanner SM, Liyanarachchi S, Fishel R, Guttridge DC. (2009) RelA/p65 functions to maintain cellular senescence by regulating genomic stability and DNA repair. *EMBO Rep* 10:1272-1278
- Watanabe, Muramatsu M-A, Hirai H, Suzuki T, Fujisawa J, Yoshida M, Arai K-I, Arai N. (1993) HTLV-I encoded Tax in association with NF-kappa B precursor p105 enhances nuclear localization of NF-kappa B p50 and p65 in transfected cells. *Oncogene* 8: 2949–2958
- Wattel E, Vartanian JP, Pannetier C, Wain-Hobson S. (1995) Clonal expansion of human T-cell leukemia virus type I-infected cells in asymptomatic and symptomatic carriers without malignancy. *J Virol*. 69(5):2863-2868
- Xiao G, Fu J. (2011) NF-kappaB and Cancer: A Paradigm of Yin-Yang. *Am. J. Cancer Res*. 1:192–221
- Xiao G, Harhaj EW, Sun SC. (2001) NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. *Mol Cell*. 7:401–409
- Xiao G, Rabson AB, Young W, Qing G, Qu Z. (2006) Alternative pathways of NF-kappaB activation: a double-edged sword in health and disease. *Cytokine Growth Factor Rev*. 17:281–293.
- Yamada Y, Tomonaga M, Fukuda H, Hanada S, Utsunomiya A, Tara M, Sano M, Ikeda S, Takatsuki K, Kozuru M, Araki K, Kawano F, Niimi M, Tobinai K, Hotta T, Shimoyama M. (2001) A new G-CSF-supported combination chemotherapy, LSG15, for adult T-cell leukaemia-lymphoma: Japan Clinical Oncology Group Study 9303. *Br J Haematol*. 113(2):375-382
- Yamaoka S, Courtois G, Bessia C, Whiteside ST, Weil R, Agou F, Kirk HE, Kay RJ, Israel A. (1998) Complementation cloning of NEMO, a component of the I $\kappa$ B kinase complex essential for NF- $\kappa$ B activation. *Cell*. 93:1231–1240
- Yamaoka S, Inoue H, Sakurai M, Sugiyama T, Hazama M, Yamada T, Hatanaka M (1996) Constitutive activation of NF- $\kappa$ B is essential for transformation of rat fibroblasts by the human T-cell leukemia virus type I Tax protein. *EMBO J*. 15:873-887
- Yin MJ, Gaynor RB. (1996) Complex formation between CREB and Tax enhances the binding affinity of CREB for the human T-cell leukemia virus type 1 21- base-pair repeats. *Mol Cell Biol* 16:3156-3168
- Yin MJ, Paulssen E, Seeler J, Gaynor RB. (1995) Chimeric proteins composed of Jun and CREB define domains required for interaction with the human T-cell leukemia virus type 1 Tax protein. *J Virol* 69, 6209-6218

- Yoshida M, Osame M, Usuku K, Matsumoto M, Igata A. (1987) Viruses detected in HTLV-I-associated myelopathy and adult T-cell leukaemia are identical on DNA blotting. *Lancet*. 1:1085–1086
- Yoshiki T. (1995) Chronic progressive myeloneuropathy in WKAH rats induced by HTLV-I infection as an animal model for HAM/TSP in humans. *Intervirology*. 38(3–4):229–237
- Yu HP, Xie JM, Li B, Sun YH, Gao QG, Ding ZH, Wu HR, Qin ZH. (2015) TIGAR regulates DNA damage and repair through pentosephosphate pathway and Cdk5 -ATM pathway. *Sci Rep*. 5, 9853
- Zane L, Yasunaga J, Mitagami Y, Yedavalli V, Tang SW, Chen CY, Ratner L, Lu X, Jeang KT. (2012) Wip1 and p53 contribute to HTLV-1 Tax-induced tumorigenesis. *Retrovirology* 9:114
- Zarnegar BJ, Wang Y, Mahoney DJ, Dempsey PW, Cheung HH, He J, Shiba T, Yang X, Yeh WC, Mak TW, Korneluk RG, Cheng G. (2008) Noncanonical NF-kappaB activation requires coordinated assembly of a regulatory complex of the adaptors cIAP1, cIAP2, TRAF2 and TRAF3 and the kinase NIK. *Nat Immunol*. 9:1371–1378
- Zhang H, Chen L, Cai SH, Cheng H (2016) Identification of TBK1 and IKKε, the non-canonical IκB kinases, as crucial pro-survival factors in HTLV-1-transformed T lymphocytes. *Leuk. Res*. 46, 37–44
- Zhao LJ, and Giam CZ. (1992) Human T-cell lymphotropic virus type I (HTLV-I) transcriptional activator, Tax, enhances CREB binding to HTLV-I 21-base-pair repeats by proteinprotein interaction. *Proc. Natl. Acad. Sci., USA* 89:7070-7074
- Zhao T, Yasunaga J, Satou Y, Nakao M, Takahashi M, Fujii M, Matsuoka M. (2009) Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-κB. *Blood* 113:2755–2764
- Zhi H, Yang L, Kuo YL, Ho YK, Shih HM, Giam CZ. (2011) NF-kB hyperactivation by HTLV-1 tax induces cellular senescence, but can be alleviated by the viral anti-sense protein HBZ. *PLoS Pathog*. 7:e1002025